MOLECULAR MECHANISMS OF CLASSICAL FEAR CONDITIONING: GABAERGIC FACTORS AND THEIR ROLE IN FEAR-RELATED NETWORK ACTIVITIES.

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von: Dipl. biol. Jorge R. Bergado Acosta

geb. am: 24.07.1976 in: Matanzas, Kuba

Gutachter: Prof. Dr. Oliver Stork

Prof. Dr. Gal Richter-Levin

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Zusammenfassung.

In der hier vorgelegten Arbeit untersuchte ich die Entstehung und Funktion rhythmisch synchronisierter Netzwerkaktivitäten im Theta Fequenzbereich, die im amygdalo-hippokampalen System während der Bildung und Expression von Furchtgedächtnissen auftreten. Der Beitrag GABAerger Mechanismen zu diesen Aktivitäten stand im Mittelpunkt dieser Untersuchungen. Hierzu setzte ich das Paradigma der klassischen Furchtkonditionierung ein, dass mir die Möglichkeit eröffnete Mechanismen der Generalisierung von Furchtgedächtnissen und ihrer Modulation durch Stress, sowie das Wirken von Gen x Umwelt Interaktionen hierbei zu analysieren. Zunächst bestimmte ich die temporale Spezifität der amygdalo-hippokampalen Thetaoszillationen in wildtyp Mäusen (Studie 1) und untersuchte die Bedeutung von Stressmechanismen für den Grad dieser Interaktion am Modell der NCAM Nullmutanten (Studie 2). Hierbei zeigte sich ein hohes Maß an Selektivität dieser Netzwerkaktivität für späte Phasen der Gedächtniskonsolidierung stimulus-spezifischer und kontextueller Furcht, sowie ihre Abhängigkeit von einer adäquaten Salienzkodierung in den konditionierten Tieren.

Auf der Basis dieser Erkenntnisse untersuchte ich dann (Studie 3) die Bedeutung der Synthese des inhibitorischen Transmitters γ-Aminobuttersäure (GABA) für die Konsolidierung und den Abruf konditionierter Furcht, sowie die hierbei auftretenden Thetaoszillationen. Zu diesem Zwecke wurden Mäuse mit genetischer Ausschaltung des Schlüsselenzyms der GABA Synthese, Glutamat Dekarboxylase (GAD)65, eingesetzt. Es gelang mir zu zeigen, das GAD65 Defizienz zu einer selektiven Störung stimulus-spezifischen Langzeit-Furchtgedächtnisses führt. Darüber hinaus konnte ich Veränderungen der rhythmischen Thetasynchronisation zwischen Amygdala und Hippokampus nachweisen, die mit der Generalisierung von Furchtgedächtnissen in GAD65 Mutanten und in übertrainierten wildtyp-Tieren assoziiert sind. Schließlich zeigte ich (Studie 4), dass das kürzlich entdeckte

Neuropeptid S (NPS), wahrscheinlich durch eine Modulation GABAerger Transmission im basolateral Subnukleus der Amygdala, kontextuelle Aspekte der Furchtgedächtnisbildung bzw. deren Abrufes beeinflussen kann. Zusammengenommen zeigen meine Daten, dass GABAerge Transmission rhythmisch synchronisierte Netzwerkaktivitäten im amygdalo-hippkampalen System in späten Phasen der Gedächtniskonsolidierung kontrollieren, und so insbesondere für die Spezifität und stressabhängige Modulation von auditorisch und kontextuell konditionierter Furcht von grundlegender Bedeutung sind.

Summary.

In the work presented here, I investigated the role of rhythmically synchronized network activity of the theta frequency range in amygdalo-hippocampus pathways during the formation and expression of learned fear. In particular, I focused on the contribution of GABAergic mechanisms to these activities. To this end I used classical fear conditioning, which provided the possibility to address mechanisms of memory generalization and stress modulation, as well as gene x environment interactions impinging on such fear memories. First, I determined the temporal specificity of the amygdalo-hippocampal theta synchronization in wild type mice (study 1) and addressed the role of stress mechanisms therein using NCAM (neural cell adhesion molecule) null mutant mice (study 2). These studies provide evidence for a specific involvement of amygdalo-hippocampal theta phase synchronization in the consolidation of cued and contextual informations at long-term stages of fear memory and their relation to the encoding of stimulus salience in the conditioned animals.

Based on these results, I then addressed the role of γ-amino butyric acid (GABA) synthesis in fear memory consolidation, retrieval and their relation to the amygdalo-hippocampal theta synchronization (study 3). Mice with targeted ablation of the key enzyme in GABA synthesis,

glutamate decarboxylase (GAD)65 were employed for this. I could demonstrate that a deficiency in GAD65 results in a selective disturbance of stimulus-specificity during long-term fear memory consolidation. Moreover, I was able to describe network activity patterns that are associated with fear memory generalization in GAD65 mutant and wild type mice. Finally, I could show (study 4) that the recently discovered neuropeptide S (NPS), likely through control of GABAergic transmission in the basolateral subnucleus of the amygdala, is capable to modulate contextual aspects of fear memory or its retrieval. Together, my data suggest that GABAergic transmission controls amygdalo-hippocampal network activities during a late phase of fear memory consolidation, and thus are critical for the specificity and stress-dependent modulation of both auditory cued and contextual fear memory.

1. Introduction.

Behavioral plasticity, that is, the capacity to modify existing behavioral patterns and to acquire new ones, allows individuals to adapt conducts according to experience. Thus, subjects must be able to detect and respond to regular (in particular, temporal) relationships between different stimuli (*classical conditioning*) or between their behavior and such stimuli (*operant conditioning*). Affective factors like emotions and motivation are irreplaceable drives that determine the relevance of every particular experience, and correspondingly control what part of this experience should be preserved. Among affective factors, fear is one of the most powerful drives to orient behavior, and in particular to avoid potentially harmful or life threatening situations. Fear reactions are innate but can also be learned, and are of outmost importance for successful adaptive behavior. In humans, learned fear can, however, lead to maladaptive consequences like phobias, anxiety or the so-called post-traumatic stress disorders (PTSD). Understanding the physiological and molecular mechanisms of fear conditioning is therefore not only important for a principle understanding of behavioral control and learning mechanisms, but can also have a decisive impact in the development of successful strategies for treating pathological fear.

1.2. Fear memory.

1.2.1. Classical fear conditioning.

In classical fear conditioning a subject is exposed to an innocuous conditional stimulus (CS+) that is coincident with a noxious unconditional stimulus (US) (LeDoux, 2000). In 1969, Leon Kamin showed that rodents do not simply learn that the conditioned stimulus precedes the unconditioned stimulus (*contiguity*) but rather that the conditioned stimulus predicts the unconditioned stimulus (*contingencies*) (Kamin, 1969; Bolles, 1971). Emotional reactions, in this learning perspective, launch the adaptive value of any set of circumstances. Individuals

will make an important advance in their capacity for self-preservation if they can anticipate and expect a traumatic situation, which entails helplessness, instead of simply waiting for it to happen (Kandel, 1999).

Learned fear can be evaluated by measuring the conditioned response (CR) during single CS presentation including: freezing, risk-assessment, suppression of operant behavior, autonomic reactivity, pain inhibition and reflex potentiation. Although these reactions are species-specific, there is a good commonality in mammals, including humans (resumed in LeDoux, 1996; Olsson and Phelps, 2007). Freezing-like behavior (complete immobilization) during fear memory retrieval generally increases with the number of CS+ / US pairings and with the intensity of the US applied during training. Nevertheless, over-training protocols can induce generalization towards a neutral conditional stimulus (CS-) that has not been paired with the US (Laxmi *et al*., 2003; Albrecht *et al*., in revision). Generalization is considered, "a cognitive act that occurs when the judgment of two different situations are likely to belong to a set of situations having the same consequence" (Shepard, 1987). This phenomenon depends on the physical properties (*salience*) of the CS, and on the strength of the US (Laxmi *et al*., 2003). Hence, it has been suggested "that although fear responses serve an evolutionary valuable function in protection from potential dangers, they may also be maladaptive in that any contextual stimulus can become associated with recurrent fear and anxiety (i.e., *generalization*)" (Garakani, *et al*., 2006).

Prolonged stress or early adverse life experiences have a number of physiological consequences that may lastingly affect fear memory formation. For example, such stressors result in increased release of corticotropin-releasing factor (CRF), the hormone released from the hypothalamus to initiate the hypothalamic-pituitary-adrenal (HPA) axis response (Carpenter *et al*., 2004). Adrenal stress hormones in turn modulate performance on various learning and memory tasks and fear memory consolidation (Roozendaal, 2000; McGaugh, 2004; LaBar and Cabeza, 2006). However, not everyone who undergoes a traumatic event

will develop a pathological fear reaction, indicating that exposure to a disturbing experience cannot entirely explain behavior (Broekman *et al*., 2007; Yehudal and LeDoux, 2007). Therefore, an interaction of genetic and environmental factors has been postulated to determine an individual's vulnerability to develop affective and anxiety disorders (Yehudal and LeDoux, 2007; Anagnostaras *et al*., 1999). Investigating principles of classical conditioning and its generalization may thus be useful for understanding the psychological and pathological basis for a variety of emotional disorders (Fend and Fanselow, 1999; Maren, 2005; Stoppel *et al*., 2006). In fact, "neural circuits underlying fear conditioning have been mapped, synaptic plasticity in these circuits has been identified, and biochemical and genetic manipulations are beginning to unravel the molecular machinery responsible for the storage of fear memories" (Maren, 2001).

1.2.2. Brain areas involved in classical fear conditioning.

The neural processing of emotion critically involves a group of subcortical nuclei phylogenetically related and identified as "limbic" structures, which organize the relationship of cognitive, motor and physiological states to a precise goal behavior (Damasio, 1995; LeDoux, 2000). There is not a real consensus about which nuclei should be included under this term (Laberge *et al*., 2006), but traditionally it is understood that old cortical structures that surround the thalamus form the limbic lobe. These structures include the cingulate gyrus and the indusium griseum; the hippocampus and dentate gyrus; the subiculum, presubiculum and parasubiculum; the entorhinal area; the prepyriform cortex; the septum; the olfactory tubercule; the medial and cortical amygdaloid nucleus; and some minor gray masses (Morgane and Mokler, 2006; Nakano, 2007). In particular the amygdaloid complex, an almond-shaped structure located bilaterally deep within the temporal lobe, appears to be decisive for the control of various positive and negative emotions, including fear memory formation (Sah *et al*., 2003).

The amygdala is structurally miscellaneous and comprises about 13 nuclei. These are distinguished on the basis of their cytoarchitecture, histochemistry, and the extensive internuclear and intranuclear connections they build (Sah *et al*., 2003). The nuclei of the amygdaloid complex are structurally and functionally heterogeneous; therefore Swanson and Petrovich (1998), recommend that they should be divided into four functional systems. In this point of view, the basolateral nucleus, which is embryologically cortical-like nuclei, receive afferents from related sources and contain cells resembling cortical neurons, will be part of the fronto-temporal system. The central nuclei, striatal in origin, contain cells morphologically similar to those in the striatum and make many connections with regions involved in autonomic control comprise the autonomic system. Finally, the cortical nuclei, and the medial nucleus, which are the major target of olfactory projections, are part of the main and accessory olfactory systems (Sah *et al*., 2003; Swanson and Petrovich, 1998). In addition, based on anterograde and retrograde innervations of the centromedial amygdaloid complex to the bed nucleus of stria terminalis (BNST) and the caudodorsal regions of the substantia inominata (ventral pallidum), some authors have argued that these regions should be termed "extended amygdala." (Shammah-Lagnado *et al*., 2000; Alheid *et al*., 1998).

The involvement of the amygdala in emotional processing has first been recognized by Klüver and Bucy (1939), who examined the behavioral effects of medial temporal lobe lesions in monkeys. These animals showed a range of effects including marked changes in emotional behavior that were described as "psychic blindness" (Ralph *et al*., 1983). These lesions were quite large and included the amygdala, hippocampus, and surrounding cortical areas. Nevertheless, these findings were later replicated with more restricted amygdala lesions in both monkeys (Meunier and Bachevalier, 2002) and humans (Hayman *et al*., 1998). In addition, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) demonstrated activation of the amygdala in healthy volunteers during retrieval of emotional memory (Büchel and Dolan, 2000; Bechara *et al*., 1995; Cahill *et al*., 1996; Canli *et*

al., 2000; Richardson *et al*., 2004). Animal studies using unit recording, brain lesions and / or pharmacological inactivation further confirmed these findings and provides evidence that the lateral amygdala (LA) is a crucial locus for auditory fear memory (Maren and Quirk, 2004).

Moreover, a general analysis in rodents with lesions and neural activity studies of substrates implicated in fear conditioning has shown that the LA, the basolateral complex (BLA) and the central nucleus of the amygdala (CeA) (LeDoux, 2000), play specific and critical roles in the acquisition and expression of auditory fear memory (Sotres-Bayon *et al*., 2004; Davis *et al*., 2003; Maren and Quirk, 2004). Sensory afferent input relays acoustic information to the LA by means of two pathways: a direct and fast thalamo-amygdala pathway and a slower pathway, which involve sensory cortex synapses before reaching the LA (Li *et al*., 1996; Quirk *et al*., 1997). The anterolateral system carries pain information to the posterior thalamus as well as to the insular cortex, and the LA receives nocieceptive information directly from these two regions (Fanselow and Poulos, 2005). From the LA, the information is send to the CeA directly, and to the BLA which is the primary nucleus in the amygdala responsible for voluntary emotional behavior based upon aversive emotional events (Armony and LeDoux, 1997; Fanselow and Poulos, 2005). The BLA integrates aversive information with context-related information from hippocampus (Pare, 2003; Maren and Fanselow, 1995) and, reciprocally, modulates hippocampal information processing.

The hippocampal formation in turn processes multimodal information concerning contexts (Atkins *et al*., 1998). Although the hippocampus does not seem to be the final deposit of affluent memory, clinical and experimental evidences indicate that it acts importantly in fear memory consolidation as well as in defining place and time of aversive situations. Support for a role in spatial control is derived principally from the observations of long-term potentiation (LTP, *see below*) in pyramidal "place cells" of the hippocampus (Shapiro, 2001; Lever *et al*., 2002). This cells respond whenever the animal is in a particular location, perceives a specific stimulus or performs a specific behavior in a particular place (O´Keefe and Burgess, 1996).

The multimodal processing of these cells fill-in to an evaluation of environments, which depends on the integration of complex polymodal sensory information. The hippocampus is also essential for temporal information processing, which can be studied in trace fear conditioning -with a brief time interval separating CS termination and US onset. Data from humans suggest that the hippocampus codes the temporal information during trace conditioning (Henke, 1997; Parkin, 1996), whereas brain regions supporting working memory processing, maintain the CS / US representation during the trace interval (Knight *et al*., 2004). Consistent with the "Hebbian" descriptions of memory formation, long-term potentiation (LTP) is recognized as a model for establishing episodic memories in the mammalian hippocampus (Bliss and Collingridge, 1993; Matthies, 1989). LTP is operationally defined as a long-lasting increase in synaptic efficacy, which follows high-frequency stimulation of afferent fibers. Activation of protein kinases required for hippocampal LPT induction (Reymann, 1988; Reymann, 1993; Reymann and Frey, 2006) are activated in the hippocampus only after associative learning (Schafe *et al*., 1999; Atkins *et al*., 1999), indicating that long-lasting endogenous plasticity in the hippocampus may be more likely observed when an arousing experience occurs in conjunction with hippocampus-dependent learning (Keeley *et al*., 2006; Diamond *et al*., 2005). Other characteristics of LTP, including its rapid induction, persistence, and correlation with natural brain rhythms, provide conditional support for this connection to memory storage (Shors and Matzel, 1997).

The hippocampus, named according to its resemblance of a seahorse in coronal slice preparations, is located bilaterally along the medial temporal lobe (Walther, 2002). It collects polymodal information from sensorial cortices and lower inputs with information about the homeostasis and general physiological arousal and plays key roles in stress modulation, memory consolidation and spatial navigation. The term hippocampal formation generally applies to the dentate gyrus (DG), the Cornu Ammonis (CA) fields (CA1, CA2 and CA3 *the hippocampus proper*), CA4 or hilus considered part of the DG, and the subiculum. The

hippocampal formation receives excitatory glutamatergic projection from the entorhinal cortex (EC) by means the perforant pathway (PP) that is distributed mainly by the DG. The second important entrance represents the system of the fimbria-fornix (FF), that provides the DG and CA fields with cholinergic, dopaminergic, and noradrenergic innervation from septum, hypothalamus and the nuclei of the reticular formation (O´Keefe and Nadel, 1978a). Injury of any of these afferents causes memory defects similar to those of the hippocampus lesion or memory deficits that accompany normal or pathological aging (Cassel *et al*., 1997; Galani *et al*., 2002). The main output pathways of the hippocampus are the cingulum bundle and the FF, which arise from field CA1 and the subiculum. In brief, the perforant input (PP) from EC layer II target granule cells and interneurons into the DG. The granule cells of the DG send their axons (mossy fibers) to CA3. Region CA3 sends fibers (schaffer collaterals) to region CA1 that receives also input from EC layer III and the nucleus reuniens of the thalamus. CA1, in turn, projects to the subiculum as the final stage in the pathway. Within the amygdaloid complex, the BLA has the most extensive interconnections with the hippocampus and their interactions may be generally involved in the conversion of short-term fear memory into long-term fear memory related to emotional arousal (Akirav and Richter-Levin, 1999; Richter-Levin, 2004). Stimulation of the BLA complex can facilitate LTP of PP inputs to the DG of the hippocampus (Nakao *et al*., 2004, Akirav and Richter-Levin, 1999; Frey *et al*., 2001).

The primary output structure of the amygdala is the CeA, which orchestrates a physiological and behavioral fear response (Davis and Whalen, 2001). Projections from the CeA to the lateral hypothalamus (LH) activate the sympathetic nervous system and lead to cardiovascular activation, pupil dilation, and increased sweating. Activation of the paraventricular nucleus of the hypothalamus through the CeA initiates the glucocorticoid response. Freezing-like behavior is mediated by projections from CeA to the periaqueductal gray (PAG), direct stimulation of which also elicits freezing or other defensive behaviors (Amorapanth *et al*.,

2008; Keay and Bandler, 2002, Keay and Bandler, 2001). Lesions of individual brain regions downstream of the CeA block specific aspects of the fear response, whereas ablation of the central nucleus itself blocks the entire response (Krout *et al*., 1998).

1.2.3. Local circuit plasticity in the amygdala during fear conditioning.

The role of the amygdala for fear memory formation is reflected in a variety of cellular and molecular processes that result in a lasting change of neural activity and network organization. As such, LTP has been described to occur not only upon stimulation of afferent fibers to the LA but also in response to behavioral training. For example, tetanic stimulation of the fast thalamic afferents induce lasting LTP-like changes on LA cells (Clugnet and LeDoux, 1990; Rogan and LeDoux, 1995) and potentiation of field potentials are observed in the LA of fear conditioned rats, recorded both *in vivo* and *in vitro* (Quirk *et al*., 1995; Rogan *et al*., 1997; McKernan and Shinnick-Gallagher, 1997). This LTP shares important characteristics with fear memory formation in particular its dependence on (N-methyl-D-aspartic acid) NMDA-type glutamate receptor activation. At thalamic inputs to the LA, an NMDA receptor independent form of LTP, carried by voltage gated calcium channels (VGCCs), can be induced through coinciding postsynaptic depolarization and pre-synaptic stimulation (Weisskopf *et al*., 1999). Molecular events of plasticity have been observed that are critical for both LTP and fear memory formation, as there are trafficking of glutamate receptors, activation of intracellular second messenger, alterations in gene expressions, and cytoskeletal rearrangements.

Synapses modify their strength by changing the number of postsynaptic receptors, and it is generally accepted that cognitive functions such as learning and memory trigger such process (Derkach *et al*., 2007; Ehninger *et al*., 2005). Glutamate receptor type 1 (GluR1) subunits of the (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) AMPA-type glutamate receptor, for example, are driven into the hippocampal neuron in response to plasticity-inducing stimuli

(Hayashi *et al*., 2000; Zamanillo *et al*., 1999). More recently, Rumpel *et al*., (2005) used a green-fluorescent-protein-tag to show that the GluR1 containing AMPA receptors (AMPARs) are inserted at thalamo-amygdala synapses after auditory fear conditioning. They further showed that preventing the insertion in ~10 to 20 % of neurons that undergo plastic change were sufficient to impair fear memory formation (Rumpel *et al*., 2005). Regulated trafficking of post-synaptic AMPARs is also involved in the expression of LTP and long-term depression (LTD) in the LA (Yu *et al*., 2008). Accordingly, genetically modified mice completely lacking the GluR1 subunit of the AMPAR (Feyder *et al*., 2007), or mice expressing GluR1 subunits with subtle mutations in phosphorylation sites that block synaptic incorporation of recombinant GluR1-receptors (Lee *et al*., 2003), show deficits in some associative forms of memory and demonstrate impaired associative fear conditioning.

Moreover, the increase of intracellular Ca^{++} through NMDA or VGCCs receptors leads to autophosphorylation of calcium / calmodulin-dependent protein kinase II (CamKII) and CamKIV (Moriya *et al*., 2000; Rodrigues *et al*., 2004; Chen *et al*., 1994; Mayford *et al*., 1996; Wei *et al*., 2002). Fear conditioning also requires the activation of protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK) (Micheau and Riedel, 1999; Sweatt, 2001; 2004; Brambrilla *et al*., 1997; Schafe *et al*., 1999; Schafe and LeDoux, 2000; Schafe *et al*., 2000; Huang *et al*., 2000; Goosens *et al*., 2000; Weeber *et al*., 2000). These effects are counteracted by the Ca^{++} sensitive protein-phosphatase calcineurin, which is increasingly expressed in the LA / BLA after extinction training (Lin *et al*., 2003a; 2003b). CamKII and IV, PKA, PKC and MAPK all converge on the calcium response element binding protein (CREB), and increased CREB phosphorylation as well as transcription from genomic CRE motifs have been observed following fear conditioning training (Impey *et al*., 1998, Stanciu *et al*., 2001; Korzus *et al*., 2004). CREB is able to induce the expression of many gene products (Lonze and Ginty, 2002) and several studies illustrate that a large number of genes are regulated at different phases during consolidation of fear memory (Helmstetter *et al*.,

2008). These include early genes like c-Fos, zif268 (Rosen *et al*., 1998; Ressler *et al*., 2002), and Arc / Arg3.1 (Ploski *et al*., 2008), other genes like α-actinin (Ressler *et al*., 2002; Mei *et al*., 2005) and GluR1 (Mei *et al*., 2005), are expressed in the amygdala shortly after training. Importantly, α-actinin and GluR1 remain at elevated expression levels at later time points (6 and 24 hours) (Mei *et al*., 2005) indicating that they are involved in the stability of the memory. Following this initial phase, a second time window of gene expression seems to be activated (Stork *et al*., 2001). For example, quantitative real time polymerase chain reaction (RT-PCR) 6 hours after fear training shows induction of structural reorganization processes (E2-ubiquitin conjugating enzyme, γ-actin, neuroligin1, proteasome subunit P31, and UDP-galactose transporter isozyme1) and myelin-associated oligodendrocytic basic protein (MOBP), neuronal cell surface protein F3, haperonin containing TCP-1, and microtubule-associated protein 4 (MAP4) (Mei *et al*., 2005; Stork *et al*., 2001).

Generation of permanent memory correlates is thought to involve the morphological modification of existing synapses and the formation of new synaptic contacts. These processes necessitate synchronized activity between molecules that regulate cytoskeletal morphology, and those that control pre- and postsynaptic cell contacts (Lamprecht and LeDoux, 2004). Adhesion molecules like integrins, cadherins, neurexin and the members of the immunoglobulin superfamily, are membrane-associated proteins that interact with the extracellular matrix and direct the tie between pre and postsynaptic membranes (Lamprecht and LeDoux, 2004; Benson *et al*., 2000). These molecules may initiate signaling pathways that couple the dynamics of extracellular with intracellular events controlling cell morphology (Dalva *et al*., 2007). The prototypical cell adhesion molecule of the immunoglobulin superfamily, the neural cell adhesion molecule (NCAM) for instance, plays a decisive role in development and plasticity of the nervous system (Bonfanti, 2006), and is involved in the mechanisms of LTP, as well as in learning and memory (Hartz and Rønn, 2008). NCAM-

associated polysialic acid (PSA) is also well situated to control and counteract different levels of stress during learning (Sandi, 2004; Cordero *et al*., 2003; Markram *et al*., 2007b).

The importance of cytoskeletal reorganization for fear conditioning has been demonstrated in various studies. Administration of actin depolymerization enhancers, cytochalasin D and latrunculin impair acquisition and extinction of contextual fear conditioning (Fischer *et al*., 2004; Mantzur *et al*., 2009). Indeed, changes in the expression of actin, α-actinin and neurofilaments as well as the serin / threonin kinase Ndr2 (a kinase that modulates actin filament dynamics) have been observed in the LA / BLA of fear conditioned animals (Stork *et al*., 2001; Ressler *et al*., 2002; Stork *et al*., 2004). Another cytoskeletal-associated protein Arc / Arg3.1 is early upregulated in the LA at both messenger ribonucleic acid (mRNA) and protein levels during fear memory consolidation (Ploski *et al*., 2008). Fear memory formation further involves activation of p160ROCK that is possibly concerned in Rho-mediated actin (Ras homologous) filament dynamics and spinogenesis (Lamprecht *et al*., 2002; Brouns *et al*., 2001). Interestingly, obstruction of the interaction between PKA and A-kinase-anchoring protein (AKAP) 150 in the LA disrupts fear conditioning (Moita *et al*., 2002), demonstrating that recruitment of PKA to the cell membrane is of critical importance. AKAPs also link small guanosine-5'-triphosphate (GTP) ases of the Rho family with the actin cytoskeleton (Diviani and Scott, 2001) and recruitment of p190RhoGap to the growth factor receptor-bound protein 2 (GRB2) complex and significant activation of downstream p160ROCK could be demonstrated after fear conditioning (Lamprecht *et al*., 2002).

Of these many fold changes, two need to be pointed out particularly in the context of the current study. First, changes in NCAM expression and function, likely related to morphological changes and cytoskeletal rearrangements. Secondly, the observation of changes in the GABAergic system upon fear conditioning (Ressler *et al*., 2002; Chhatwal *et al*., 2005; Heldt and Ressler, 2007) and the sensitivity of fear memory and LTP to disturbances of GABAergic function in the amygdala and hippocampus. For instance,

microinjections of muscimol, an γ-aminobutyric acid (GABA) $_A$ receptor agonist in the BLA reduced expression of early genes (Arc and c-Fos) in the hippocampus after context exposure, context plus shock, but not after shock alone (Huff *et al*., 2006). This approach has critically initiated the study presented here, as a regulation of the key enzyme in GABA synthesis, glutamic acid decarboxylase (GAD), was observed in the amygdala upon fear conditioning (Pape and Stork, 2003).

1.3. GABA interneurons.

1.3.1. GABA synthesis, receptors and reuptake.

Two main types of neurons have been described in the LA / BLA amygdala complex. Pyramidal, or "class I" cells that comprises 70 % of the whole population and have pyramidal-like somata; and stellate or "class II", that contain two to six primary dendrites forming a relatively spherical field. The populations they form are heterogeneous and have been divided according to their dendritic trees into multipolar, bitufted, and bipolar cells. These neurons are mostly GABAergic and represent a group of local circuit interneurons (Millhouse and DeOlmos, 1983; McDonald, 1983). GABA is generated in these cells by the enzyme glutamic acid descarboxylase, which catalyses the decarboxylation of glutamic acid.

Two major isoenzymes of GAD, named GAD65 and GAD67 according their approximate molecular weight of 65±4 and 66±6 kDa respectively, have been identified in human brain (Bu *et al*., 1992; Soghomonian and Martin, 1998). GAD65 and GAD67 homologs are expressed in birds, reptiles, and fish; a single GAD cDNA (complementary DNA) with equal similarities to both vertebrate GAD forms is found in the protochordate (Bosma *et al*., 1999). GAD65 is preferentially localized in axon terminals (Esclapez *et al*., 1994), more strongly membrane associated and more often exists in an inactive apoGAD form (lacking the cofactor pyridoxal phosphate) compared with the GAD67 isoenzyme (Kaufman *et al*., 1991; Fenalti *et*

al., 2007) suggesting that GAD65 might preferentially synthesize GABA for vesicular release and that GAD67 may be preferentially involved in synthesis of cytoplasmic GABA (Houser and Esclapez, 1994; Esclapez *et al*., 1994; Bowers *et al*., 1998).

GABA binds to two main receptor types that are recognized in terms of different pharmacological, biochemical and electrophysiological properties (Browner *et al*., 1981). The ionotropic $GABA_A$ receptors consist of 5 subunits placed around a central pore that create a chloride ion channel, and Cl^- influx is thought mediate fast membrane hyperpolarization (Olsen *et al*., 1991). There are 16 different subunits called α1-6, β1-3, γ1-3, δ, ε, ρ1-3 and θ. This repertoire allows more than 2000 combinations, with the major being α1β2 / 3γ2, α2β3γ2 and α3β3γ2. The significance of $GABA_A$ in modulation of fear, anxiety and acute stress is remarkable, as this receptors bears allosteric binding sites for benzodiazepines, barbiturates, and neurosteroids (Woo and Lu, 2006; Möhler, 2006). The $GABA_B$ receptor is localized in both excitatory and inhibitory synapses, it is built from two related seven-transmembrane domain receptor subunits that are linked to G-protein and second messenger cascade. $GABA_B$ receptor agonists may be useful for the treatment of pain and drug-dependence, whereas antagonists emerge positive as cognitive enhancers and antiabsence epilepsy agents (Kornau, 2006). GABA reuptake is mediated in neurons and glial cells by different transporters GAT1, GAT2, GAT3, and BGT1 (Borden, 1996). Increased GABA at the synaptic cleft in mice with point mutation of the GAT1 transporter, lead a phenotype with decreased level of depression and anxiety like behaviors (Liu *et al*., 2007).

1.3.2. GABA interneurons are critical in fear memory.

The amygdala is the main target in the regulation of fear and anxiety behaviors and GABA modulation in this region appears to be critical to satisfy this functions (Paulus *et al*., 2005; Zhang and Cranney, 2008). One main finding was that lesions of the BLA block the amnestic

effects of diazepam, and diazepam-induced anterograde amnesia. (Tomaz *et al*., 1991; 1992). Benzodiazepines are the most used prescription to treat several anxiety disorders, and this effect is mediated by $GABA_A$ receptors (Mandrioli *et al*., 2008; Möhler, 2006). The diversity of this receptor has functional significance therein (Lorez *et al*., 2000), as the α1 subunit seems to mediate sedation while the α2 subunit have been found to mediate anxiolysis (Möhler, 2006; 2007). Immunohistochemical and electrophysiogical studies corroborated this finding and showed that α2 containing GABA receptors are concentrated in the BLA and CeA where they control inhibitory post-synaptic currents (IPSC) amplitude induced by diazepam (Marowsky *et al*., 2004). Mutant mice with point mutation in the α2 subunit showed also insensitive to diazepam (Löw *et al*., 2000).

The role of GABA transmission in fear memory formation has been studied in several manners by using $GABA_A$ receptor antagonist or agonist injected before or after CS / US pairing. Pre-training infusions of the $GABA_A$ agonist muscimol into the LA / BLA complex eliminated acquisition of fear memory, whereas post-training infusions had no consequences (Wilensky *et al*., 1999; 2000). Injection of FG7142 (a partial inverse agonist at the benzodiazepine $GABA_A$ receptor) prevent the animals to reduce fear induced via overexpectation training, but had no effect on responding to a CS paired with a low magnitude US (Garfield and McNally, 2009). Withdrawal of chronically administered diazepam increased freezing to both associative and nonassociative contexts, but this increase was greatest in the paired context group (Isoardi *et al*., 2004). These results correspond to the notion that the formation of fear memory appears to be associated with a depression of GABA function (Muller *et al*., 1997; Wilensky *et al*., 1999). In fact conditioned fear in mice is associated with a reduction of extracellular GABA levels in the amygdala (Stork *et al*., 2002). Similarly, heterozygous null mutant mice for the γ2-subunit of the $GABA_A$ receptor, with reduced clustering of $GABA_A$ receptors and deficits in GABAergic transmission, display an enhanced conditioning to threat cues (Crestani *et al*., 1999).

Indeed, reduction in gene expression of GABA related factors have been seen in the amygdala after fear conditioning. Gephyrin, a $GABA_A$ receptor clustering protein is significantly downregulated in the BLA after fear acquisition in rats at mRNA as well as protein levels (Ressler *et al*., 2002; Chhatwal *et al*., 2005). Gephyrin promotes the stabilization of $GABA_A$ receptors interacting with the γ2 subunit of the $GABA_A$ receptor (Kneussel and Loebrich, 2007). In agreement with this, autoradiographic analyses of benzodiazepine-sensitive $GABA_A$ receptor binding showed decreases of $GABA_A$ receptors at the cell surface after fear acquisition (Chhatwal *et al*., 2005). Decrease in the mRNA levels of α1, α5 and GAD67, were observed by *in situ* hybridization in the amygdala after conditioning (Heldt and Ressler, 2007) and reduction of GAD65 expression was also detected in the BLA 24 hours after training (Pape and Stork, 2003).

The role of GAD65-mediated GABA synthesis in the control of emotional behavior and synaptic transmission has been further investigated in two different lines of mice lacking this enzyme (Kash *et al*., 1997; Asada *et al*., 1996). Both genotypes are susceptible to seizures, have altered responses to anxiolytics and show increased anxiety (Asada *et al*., 1996; Kash *et al*., 1999; Stork *et al*., 2000). Specifically, $GAD65^{-/-}$ knockout mice displayed increased anxiety-like behavior in light-dark avoidance test, reduced intermale aggression, and reduced immobility induced by forced-swimming (Stork *et al*., 2000). Moreover, these mice show reduced freezing to both cue and contextual memory retrieval but increased flight response (Stork *et al*., 2003). Basal synaptic transmission in $GAD65^{-/-}$ mice (Kash *et al*., 1997) appears to be regular in the absence of stimulation but during continuous stimulation a striking deficiency in transmitter release is revealed (Tian *et al*., 1999). Interestingly, for startle reflex the same mutants respond normally during baseline and habituation but have deficits in prepulse inhibition (Heldt *et al*., 2004).

Furthermore, induction of LTP in amygdala excitatory cells depends on the strength of the local inhibitory network (Marsicano *et al*., 2002; Shumyatsky *et al*., 2002). Although GABA

cells comprise about 10 to 20 % of the neuronal population in the lateral and basolateral nuclei of the amygdala, they set the output of projection neurons (Rainnie *et al*., 1991) and direct sensory evoked responses and network synaptic activity (Lang and Paré, 1997; Szinyei *et al*., 2000). Inhibitory interneurons in the LA are capable of long-lasting potentiation that is not synapse-specific and might explain the increased synchronization of activity between neurons in the amygdala upon fear conditioning (Bauer and LeDoux, 2004). At first Mahanty and Sah (1998), reported an NMDA-receptor independent long-term potentiation to inhibitory interneurons in the basolateral amygdala. These authors demonstrated that glutamatergic synaptic transmission onto interneurons could produce Ca^{++} entry through AMPA-type receptors. In contrast Szinyei and coworkers (2003), showed that GABAergic interneurons in the LA and BLA also express functional NMDA-receptors that participate in basal synaptic transmission at both thalamic and cortical inputs. Therein the NMDA receptor 2B (NR2B) subunits appeared to be significant for NMDA receptor-mediated signaling (Szinyei *et al*., 2003).

1.4. Network activities involved in fear conditioning.

1.4.1. Oscillatory network activities and GABA.

One remarkable feature of several brain regions is the ability to generate oscillatory activity. Network oscillations covers from slow frequency in the delta (0.5-3 Hz) and theta (3-8 Hz) ranges, to fast oscillations in the gamma (30-90 Hz) and ultrafast (90-200 Hz) ranges (Buzsáki and Draguhn, 2004). The generation of network oscillations needs regular and synchronized neuronal activity (Ritz and Sejnowski, 1997). One single neuron firing regularly will activate many postsynaptic cells at that time. If all this cells fire together periodically the signal will be amplified in a large population (Llinas, 1988). This creates temporal windows of increased and reduced activity in those regions, which can be easily recorded by extracellular electrodes

in behaving animals (Buzsáki *et al*., 1983). The effects of synchronized brain activity are compatible with the formation and activation of dispersed neuronal circuits that may support sensory information processing (Bland, 1986), mnemonic functions (Landfield *et al*., 1972), memory formation (Klimesch, 1996; Jensen, 2001), and expression of behavior (Buzsaki *et al*., 1983; Klimesch *et al*., 1996; Klimesch *et al*., 1997).

GABAergic inhibition controls spike timing of pyramidal cells plays a fundamental role in the regulation of oscillatory network activity (Gonzalez-Burgos and Lewis, 2008; Buzsáki and Chrobak, 1995). Interneurons are especially diverse and can be classified among other features according to their intrinsic membrane responses, and the specificity of the input and output targets (Freund and Buzsaki, 1996). Some interneurons (of slow spiking frequency) in stratum lacunosum moleculare, the axons of which terminate on the distal dendrites of excitatory neurons shows inclination for generating theta-frequency (Chapman and Lacaille, 1999). A different hippocampal interneuron population, which targets the perisomatic region and presents different intrinsic membrane properties (fast spiking frequency) support outputs in the gamma-frequency range (Pike, 2000). *In vitro* studies provide more evidence showing that the somatic compartments of pyramidal cells receive inhibitory postsynaptic potentials (IPSP)s at gamma frequencies coincident with the gamma-frequency action potential generation (Whittington *et al*., 2000). However, the distal dendritic compartments receive mainly theta frequency IPSPs (Gillies *et al*., 2002). Hippocampal projection cells then receive different gamma and theta inhibitory postsynaptic current oscillations in soma and dendrites, conferring local temporal control of afferent inputs in different parts of the pyramidal cell (Whittington and Traub, 2003). In functional terms it has been suggested that interneurons targeting perisomatic regions are affecting somatic action potential generation, whereas those terminating more distally may modulate the induction of synaptic plasticity (Miles *et al*., 1996).

1.4.2. Role of theta in behavior and memory in general.

Among many types of brain oscillation, rhythms in the theta frequency range are important for a wide variety of behaviors and information processing. (Klimesch *et al*., 1996; Buzsaki, 2002; Buzsaki *et al*., 2003). Theta rhythm is a synchronous electroencephalogram (EEG) activity that was described as one of the essential patterns recorded from the human scalp in Hans Berger's seminal papers on electroencephalography (Berger, 1938). The demonstration that a similar pattern of activity can also be recorded from the hippocampus in animals (Green and Arduani, 1954), and evidences suggesting a relationship between hippocampal theta and memory functions (Grastyan *et al*., 1959) raised the interest of experimental brain researchers on this intriguing pattern of neuronal activity. Theta-frequency stimulation has been used for LTP induction (theta burst stimulation), more convincingly than the alternative high frequency (100 pulses of 100 Hz train stimulation for 1 s), which occurs rarely in the brain (Shors and Matzel, 1997). For example, theta burst stimulation to the PP of the hippocampus induced LTP in DG granule cell synapses (Maroun and Richter-Levin, 2002), and induces a local form of late phase LTP in the CA1 region of the hippocampus (Huang and Kandel, 2005).

Theta waves have been recorded from all the hippocampal formation subfields, in particular from the CA1 and the DG. It consists of regular, quasi-sinusoidal waves in the range from 4-12 Hz (Bland, 1986). The study of behavioral and pharmacological correlates of hippocampal theta showed that there are two different types of theta activity: a movement related type 1 theta (theta 1), and an immobility related type 2 theta (theta 2) (Bland, 1986). The neural and neurochemical bases of theta 1 are still not completely established although there are indications that serotonergic activity from the raphe nucleus is a main component of this system (Gemma *et al*., 1999). On the contrary, theta 2 has been clearly identified as a cholinergic rhythm (Bland, 1986) with the medial septal-basal forebrain integrating limbic, hypothalamic and reticular influences, and acting as a pacemaker for hippocampal theta 2

(Bland *et al*., 2006; Jackson and Bland, 2006; Pan and McNaughton, 2002). The prefrontal cortex is phased-locks to the hippocampal theta rhythm in freely behaving rats during spatial working memory tasks (Siapas *et al*., 2005; Jones and Wilson, 2005), and alterations in hippocampal place cells occur specially after contextual fear conditioning (Moita *et al*., 2004).

1.4.3. Rhythms in the hippocampus.

It has been suggested that theta oscillations represent the "on-line" state of the hippocampus (Buzsaki, 2002) for information processing. Neural activity often becomes rhythmic during mental processing (McNaughton *et al*., 2006) leading to the suggestion that theta rhythm allows the hippocampal formation to alternate rapidly between conditions that promote memory encoding and conditions that promote memory retrieval (Manns *et al*., 2007). A functional relationship between theta and memory functions is well supported by experimental confirmation using different learning tasks (Bland *et al*., 2007; Kahana *et al*., 2001; Olvera-Cortes *et al*., 2002; Olvera-Cortes *et al*., 2004; Seager *et al*., 2002), and evidences suggest that a similar relationship occurs in humans (Burgess and Gruzelier, 1997).

Hippocampal pyramidal cells are functionally heterogeneous in relation to the generation of theta-band oscillation and synchrony. In field CA1 pyramidal cells form theta-related subsets of phasic theta-ON cells and tonic theta-ON cells and non-theta-related subsets of simple spike discharging cells, complex spike discharging cells and silent "cells" (Bland *et al*., 2005), which are recruited by medial septum cells during the transition from irregular activity to theta (Bland *et al*., 1999; Garner *et al*., 2005). The pacemaker role of the medial septum in theta generation does not mean that acetylcholine (ACh) is the only transmitter involved in modulation. There is convincing evidence showing that GABAergic synapses towards hippocampus and GABAergic neurons within the hippocampus (Kopp *et al*., 2004; Yoder and Pang, 2005) play a role in the generation of theta. Rhythmically discharging basal forebrain units comprise cholinergic, GABAergic, and putative glutamatergic cells (Manns *et al*., 2003).

Cholinergic and GABAergic medial septal afferents contribute to hippocampal theta activity in part by actions on local interneurons (Chapman and Lacaille, 1999), which are main targets of this innervation (Deller *et al*., 1999). GABAergic neurons in the medial septum, which are known to selectively innervate hippocampal interneurons, are in a position to induce rhythmic disinhibition in the hippocampus and other theta-related subcortical areas (Borhegyi *et al*., 2004). On the other hand GABAergic projecting neurons from the hippocampus to the medial septum and other cortical areas contribute to coordinate oscillations in the theta band (Goldin *et al*., 2007; Jinno *et al*., 2007; Manseau *et al*., 2008). This feedback circuit seems to provide the mechanism for the rhythmic suppression of interneuronal activity in the hippocampus, which is observed as GABAergic-mediated theta activity (Denham and Borisyuk, 2000).

1.4.4. Propensity of amygdala neurons for rhythms.

Thalamic and cortical afferent fibers to the LA / BLA are monosynaptically and symmetrically connected to principal cells, and to GABAergic local circuit neurons (Szinyei *et al*., 2000). Under continuous depolarizing stimuli, pyramidal cells exhibit intrinsic oscillatory activity at theta frequencies that seems to be controlled significantly by intracellular cyclic adenosine monophosphate (cAMP) (Pape *et al*., 2005). In fact most projections cells into the LA and BLA are able to produce rhythmic-oscillatory activity with low-threshold and high-threshold at 4-10 Hz frequencies over a large series of membrane potentials. The low-threshold oscillation is mediated through a persistent sodium current, which periodically interacts with a specific type of potassium current, while the high-threshold oscillation is calcium dependent, which, in turn activates a calcium-dependent potassium channels (Pape and Driesang, 1998; Pape *et al*., 1998). The amygdala integrates different sensorial stimuli and modulates declarative memories of emotionally arousing events in other brain regions (Cahill and McGaugh, 1998). Thus oscillatory activity in amygdala

neurons could be responsible for such interactions and in the case of fear, implicated in its consolidation (Paré *et al*., 2002).

Interestingly, perirhinal and amygdala theta rhythm is phase locked to entorhinal and thus to hippocampal theta (Collins *et al*., 1999; Collins *et al*., 2001). Although direct connections between amygdala and hippocampus have been described (Pitkänen *et al*., 2000), lesion studies of perirhinal cortex demonstrated impairment of contextual fear conditioning suggesting that this region receives hippocampal information that is later relayed to the amygdala (Corodimas and LeDoux, 1995; Sacchetti *et al*. 1999). Indeed, these both regions receive neocortical inputs indirectly via perirhinal cortex (Suzuki, 1996). Functionally, amygdala oscillations at the delta and theta frequency range are coupled to the perirhinal cortex. Theta activity is also observed in the amygdala of the cat during periods of forceful arousal as well as during rapid eye movement (REM) sleep (Paré and Gaudreau, 1996; Paré and Collins, 2000). REM sleep periods have been linked to synaptic plasticity and memory consolidation (Graves *et al*., 2001; Benington and Frank, 2003). Direct projections from the endopiriform nucleus to the BLA have been also described and stimulation of this nucleus can evoke excitatory postsynaptic potentials in the BLA (Behan and Haberly, 1999; Gean and Chang, 1992).

In contrast with the hippocampus where neurons are organized in layers, in the LA / BLA complex, projection cells and various subtypes of local-circuit GABAergic neurons are intermingled (McDonald, 1992), complicating the studies of GABA inhibition in the generation of oscillatory activity in this nucleus. However, it is possible to distinguish between interneurons and projections cells according to their differences in firing rates and action potential duration (Likhtik *et al*., 2006). *In vivo* extracellular recording of the cat LA amygdala by Lang and Paré (1997) shows constant IPSP responses no matter of the stimulation site. Interneurons in the BLA could be also classified in terms of distinct neuropeptides and calcium-binding proteins they express (Mascagni and McDonald, 2003;

McDonald and Mascagni, 2002; McDonald and Mascagni, 2001). *In vitro,* paired recording from interneuron / interneuron and from interneuron / principal neuron in cells expressing green fluorescent protein (GFP) under parvalbumin promoter control, demonstrated electrical coupling between interneurons, whereas interneuron / principal neuron pair recordings exhibit large heterogeneity. These data suggest that interneurons in the BLA provide a powerful inhibition that operates to inhibit firing of pyramidal cells during cortically driven oscillations (Woodruff and Sah, 2007a; Woodruff and Sah, 2007b). These few studies at least indicated that GABA interneurons importantly contribute in the generation and the control of amygdala oscillations.

1.4.5. Rhythms in the amygdala during fear memory.

It has been suggested that theta waves in the temporal limbic lobe correspond to periods of high emotional arousal (Vinogradova, 1993). It is also accepted that synchronized network activity is fundamental for synaptic plasticity and memory formation (Bliss and Collingridge, 1993). Oscillatory activity at the theta frequency range into the amygdaloid complex moreover have been linked with the formation and consolidation of emotional memories (Pelletier and Paré, 2004). For instances, cats implanted with multiple microelectrodes in the BLA learned to predict a foot shock administered 5 min. after a tone. After several CS / US presentations animals learned to anticipated the US showing increases in blood pressure in conjunction with increases in the firing rate of BLA neurons that become synchronized at theta 4-7 Hz frequency (Pare and Collins, 2000). Indeed, simultaneous field potentials recordings between CA1 and LA demonstrated theta synchronization in response to fear-conditioned auditory stimulus presentation (Seidenbecher *et al*., 2003). Theta synchronization in amydalo-hippocampal pathways during retrieval of conditioned fear may reflect a physiological mechanism associated to long-term fear memory in this pathway (Pape *et al*., 2005). Several studies have shown increased plasticity in amygdala and hippocampus after

fear learning. In addition theta expression could be a signal of anticipatory behavior observed in conditioned animals. Therefore it may direct the generalization of fear behavior and control balance between cued and contextual fear memory retrieval (Laxmi *et al*., 2003; Seidenbecher *et al*., 2003).

1.7. Aim of this study.

In this study I was prompted to investigate the role of GABAergic inhibition in specific aspects of the formation and retrieval of fear memory. To this end I used a very well established animal model of implicit memory formation, classical fear conditioning, which further allowed me to address mechanisms of memory generalization and associated network activities. The paradigm also offered the possibility to elucidate gene x environmental interactions and stress effects, by applying different training intensities to wild type and / or different mutants mice. In the first two studies presented in this dissertation, I determined the temporal characteristics of fear memory-related theta oscillation in the amygdala-hippocampal pathway and their relation to stimulus salience, using wild type and NCAM null mutant mice. Then, I specifically addressed the role of GABA synthesis in fear memory consolidation and generalization and their relation to the amygdalo-hippocampal theta synchronization, using mice with targeted ablation of the key enzyme in GABA synthesis, GAD65. Finally, I began to characterize effects of GABA network modulation in the BLA through neuropeptide S (NPS)-mediated stimulation of the endopiriform cortex, and its role in fear conditioning.

2. Material and methods.

2.1. Subjects.

Adult male mice were used for experiments, they were keep in our animals facility (S1 level) with food and water *ad libitum,* at 12 hours light / dark cycle (light on at 7 pm). Animals were housed individually in ventilated cages for at least 7 days before stared experiments in order to keep individuality. All studies were conducted in accordance with the European and German regulations for animal experiments and approved by the *Landesverwaltungsamt Sachsonia-Anhalt* (permission NR 42502 / 2-441 UNI MD).

In study Nr.1

Eight-to-twelve weeks old male C57B / 6 mice (M&B Taconic, Berlin, Germany) were used. They were purchased at an age of six to seven weeks and kept in groups of 3-6, and housed individually after surgery.

In study Nr.2

NCAM mutants were obtained from heterozygous breeding. Genotypes were determined by multiplex polymerase chain reaction (PCR) on genomic DNA derived from tail cuts shortly after weaning. Training and testing of animals were always done during the dark cycle between 10:00 am and 6:00 pm in the training apparatus described below.

In study Nr.3

Eight-to-ten week old male C57BI / 6 (M&B Taconic, Berlin, Germany) and mutant mice lacking the gene coding for the GABA-synthesizing enzyme GAD65 (Asada *et al*., 1996), were used in these experiments. Homozygous ($GAD65^{-/-}$) and heterozygous ($GAD65^{+/-}$) GAD65 mutants and their wild type littermates ($GAD65^{+/+}$) were on a C57Bl / 6 genetic

background (>10 generations of backcross) and obtained from $GAD65^{+/-}$ X $GAD65^{+/-}$ breeding. Genotypes were determined with allele-specific PCR at the time of weaning.

In study Nr.4

Ten-to-fourteen week old male C57BL / 6 BomTac mice (M&B Taconic, Berlin) were used.

2.2. Behavioral analysis.

The training apparatus (TSE, Bad Homburg, Germany) comprised of a 36 cm x 21 cm x 21 cm light-blue acrylic glass arena with a grid floor for delivery of electric foot shocks. It was enclosed in an isolation cubicle containing a speaker, a ventilation fan providing fresh air, and background noise of 70 dB sound-pressure level (SPL). As measures of fear memory, freezing (complete immobilization except for respiratory movements) and risk-assessment behavior (overt watching, stretched attending) were evaluated off line, using a time line version of the public domain program *Wintrack* provided by D. Wolfer (Univ. Zurich).

In study Nr.1

Seven days after surgery animals underwent differential auditory cued fear conditioning in which behavioral and electrophysiological responses to the conditioned (CS+) and neutral acoustic stimuli (CS-) were recorded. CS- and CS+ (2.5 kHz or 10 kHz, 85 dB SPL, for 10 s) were counterbalanced between subjects in order to avoid any frequency-related bias, and inter-stimulus intervals (ISIs) were randomized between 10 s and 40s. Moreover, two different contexts (the “neutral context” and the “shock context”) were used in order to determine the degree of background contextual conditioning and associated theta activity.

On days 1 and 2, mice were pre-exposed (one set per day) to the neutral context for 280 s; with 6 neutral acoustic stimuli (CS-), followed 1 h later by the shock context (280 s; 6 neutral acoustic stimuli, CS-), followed 2 h later by the neutral context (280 s; 6 CS-), followed 10

min later by the shock context (280 s; no acoustic stimuli). On day 3 (training day), mice were placed in the neutral context and presented with four CS- and one pre-exposure to the CS+. Behavioral and electrophysiological recordings during the first CS- and the CS+ pre-exposure were used as baseline values (for cued memory).

One hour later, in the shock context, three CS+ each co-terminated with an aversive unconditioned stimulus (US; scrambled foot shock, 0.4 mA, 1 s) were presented. Behavioral and electrophysiological recordings immediately prior to conditioning were used as baseline values (for context memory). Two hours later mice were returned to the neutral context and confronted with 2xCS- / CS+ (no shock) / 2xCS-. Behavior and electrical activity (local field potentials) were recorded during the first CS- and the CS+ as a measurement for auditory cued memory (2 h values). Ten minutes later mice were re-introduced to the shock context for 3 min to determine background contextual memory. On day 4, auditory cued memory was assessed again (24 h value) in the neutral context using a set of 3xCS- / CS+ (no shock) / CS- with a randomized ISI of 10 s to 40 s. One hour later mice were studied in the shock context for background contextual memory.

For foreground contextual conditioning animals were trained in the shock context alone without any pre-exposure to the acoustic stimuli. In each of the three training sessions 6 USs were presented (scrambled foot shock, 0.4 mA, 1 s) at interstimulus intervals of 20 s. Animal behavior and local field potentials were recorded in the shock context before training (baseline), as well as 2 minutes, 30 minutes and 2 h after training. On the second day, data were recorded in a single 3 min exposure to the shock context (24 h values).

In study Nr.2

$NCAM^{-/-}$ and $NCAM^{+/+}$ mice received auditory fear conditioning with a highly stressful US intensity (overtraining) with 10 CS+ / US pairings at 0.6 mA foot shock for two day. Since our behavioral experiment showed that with pre-adaptation $NCAM^{-/-}$ mice can overcome the

fear memory deficits under normal training with contextual pre-exposure, all animals were habituated for two days with two minutes context pre-exposure and received six neutral auditory stimuli CS- (2.5 kHz, 85 dB SPL, 10 s, 20 s ISI.) each day. During retrieval the animals were exposed to the training context alone for 2 min, followed by a block of 4 CS- and 4 CS+, with inter-stimulus-intervals of 20 s each.

In study Nr.3

On the first day of the experiment, the animals were exposed twice to the training apparatus and a set of 6 acoustic stimuli (2.5 kHz sinus tone, 85 dB SPL, 10 s with 20 s, ISIs) serving as the CS-. On the following day, after 2 min of habituation to the apparatus animals were fear conditioned with 3 conditional stimuli (CS+; 10 kHz sinus tone, 85 dB SPL, 10 s with 20 s ISIs) that each co-terminated with an unconditional stimulus (US; 1 s scrambled foot shock, 0.4 mA). Overtraining consisted of two training sessions each containing 10 CS+ / US pairings at an increased US intensity (0.6 mA), a protocol known to induce significant generalization to the CS- and to background context (Laxmi *et al*., 2003). Fear memory retrieval was tested either at short-term (30 min post training) or long-term (24 h post training or 14 d post training) time points in a neutral context, consisting of a white acrylic 26 x 20 x 14 cm “standard cage” and saw dust filling, placed in a different room without an isolation cubicle. A fourth group of animals was tested for long-term fear memory (24 h post training) in the conditioning context, to address the potential context dependency of generalization. In all retrieval tests, after 2 min of habituation, animals were confronted with a set of 4 CS- and subsequently with 4CS+ with 20 s ISIs.

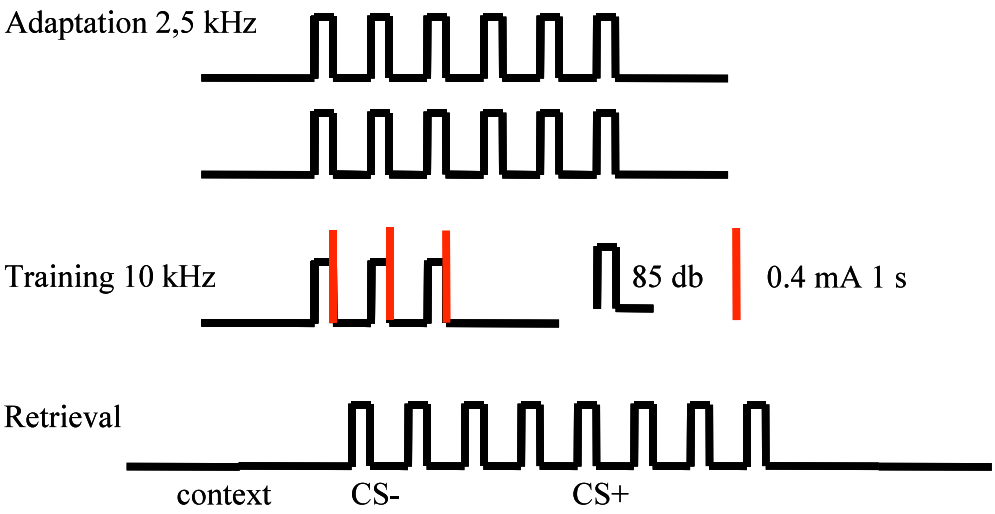


Fig 2.1. **Experimental design** used in study Nr. 3 to conditioned the mice, in the rest of the experiments similar protocols with some modification were used.

In study Nr.4

A set of 6 acoustic stimuli (2.5 kHz sinus tone, 85 dB SPL, 10 s with 20 s, ISIs) was delivered to serve as auditory control stimulus (CS-). On the following day, after 2 min of habituation to the apparatus, animals were fear conditioned with 3 conditioned stimuli (CS+; 10 kHz sinus tone, 85 dB SPL, 10 s with 20 s ISIs.) that each co-terminated with an unconditional stimulus (US; 1 s scrambled foot shock, 0.4 mA). After recovery from surgery for one week, mice were injected with 0.1 nmol NPS, 0.01 nmol NPS or vehicle (saline) at a volume of 0.3 µL per animal. 15 min after injection, general anxiety level was assessed in an elevated plus maze (arm length 60 cm large, arm width 6 cm, wall height 15 cm, elevation 40 cm) in a single 5 min session under low-light conditions (<200 Lux). Exploration of open and closed arms was evaluated using a video tracking software (ANY-maze, Stoelting, Wood Dale Il). 10 min later retrieval of conditioned fear memory was investigated. Animals were exposed to the training context for 2 min and then to sets of each 4 CS- and 4 CS+ with stimulus intervals of 20 s each.

2.3. In vivo electrophysiological analysis.

Animals were implanted with electrodes under Phenobarbital anesthesia (50 mg / kg ip) using a small animal stereotactic device (World Precision Instruments, Inc. Sarasota, FL USA). Reference and ground silver electrodes were implanted close to the midline over the nasal and cerebellar regions, respectively. Stainless steel electrodes were positioned unilaterally into the left hemisphere at the coordinates AP -1.94 mm., ML 1 mm, DV 1.25 mm from bregma and AP -2.06 mm, ML 3.25 mm, DV 4.2 mm. from bregma, aiming at the CA1 hippocampal region and the lateral amygdala, respectively (Fig 2.2.) (Seidenbecher *et al*., 2003). The electrode ensemble was fed through a rubber socket and fixed on the skull with dental cement. The prepared animals were allowed to recover for 3-4 days before behavioral training commenced. Before testing, a plug was connected to the implanted socket under a low and brief dose of an inhalable anesthetic (Isofluran) to allow the simultaneous recording of the amygdalar and hippocampal field potentials. Animals were tested with 4 CS- and 4 CS+. The amplified signals were band-pass filtered at 0.3 and 30 Hz with a 1 kHz sampling rate, fed to an A-D converter (CED, Sci. Prov., Cambridge) and digitally stored together with markers of freezing and risk assessment behavior on a PC for off-line analysis using Spike 2 software. After completion of the experiments, animals were sacrificed with an overdose of Pentobarbital (200 mg / kg ip) and the locations of the electrode tips were verified histologically in accordance with a mouse brain atlas (Paxinos and Franklin, 2001).

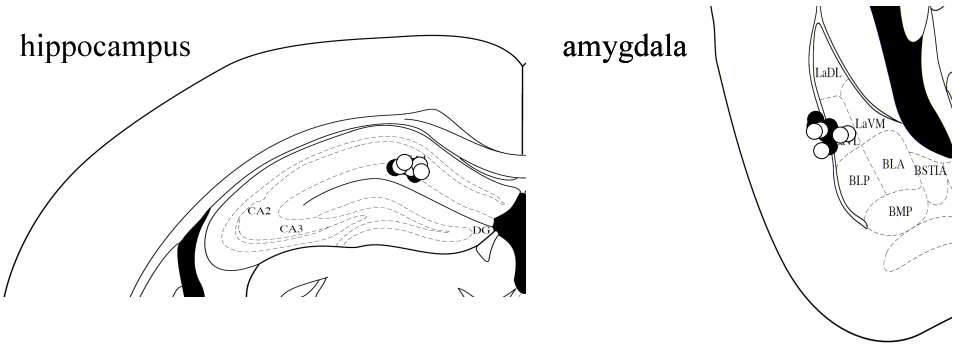


Fig. 2.2. **Locations of the electrode tips**. Position of electrodes in CA1 hippocampus region and LA / BLA amygdala nucleus for the $NCAM^{+/+}$ (black circle) and $NCAM^{-/-}$ (white circle) mice. Illustrations modified from Paxinos and Franklin 2001.

In study Nr. 1

Field potential wave forms were recorded with a differential amplifier (Science Products DPA-2F), band pass filtered from 1 to 30 Hz, transformed by an A / D interface (CED Power 1401, sampling rate: 1 kHz) and stored on-line on a personal computer. Field potential waveforms were analyzed using Spike2 and Matlab. Color-coded power spectra were calculated using a custom Matlab routine computing the windowed Fourier Transform with a fixed window size of 1.6 seconds.

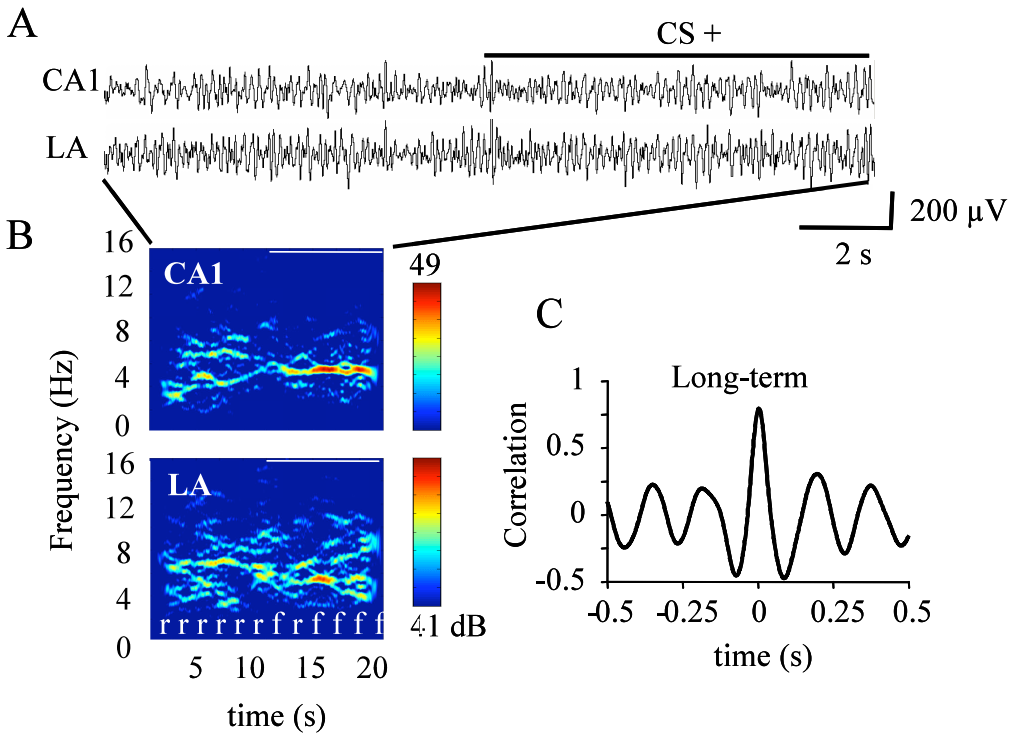


Fig 2.3. **Neural activity in CA1 and LA after cued fear conditioning**. (A) Representation of original traces of field-potential recordings before and during presentation of CS+ (black bar above the traces) at 24 h after training. (B) Color-coded power spectra of the original traces, letters indicate behavioral recording (f, freezing; r, risk-assessment). Data displayed are given by the modulus of the Fourier-coefficients over time and frequency which represents the power of a given frequency at a given time. (C) Cross-correlation theta activity in CA1 and LA during retrieval of fear memory at 24 h.

To determine stimulus-related activity, all of the 10 s periods of CS- and CS+ presentations were considered, and 3-4 s freezing periods were identified for the analysis of behavior-related correlations. For contextual conditioning, the first 40 s of recording in the retrieval context were divided into four separately analyzed 10 s periods. Since the behavioral response was constant over those four 10 s periods, only the first 10 s period was used for the illustration of freezing behavior and freezing associated theta frequency synchronization.

Cross-correlograms were averaged across animals after alignment to the maximal positive peak.

Study Nr. 2

$NCAM^{-/-}$ and $NCAM^{+/+}$ mice underwent overtraining as described above. Using a swivel commutator, field potentials of LA and CA1 were then recorded in the freely behaving animal undergoing a standard retrieval session. Amplified signals, band-pass filtered at 0.3 and 30 Hz with a 1 kHz sampling rate, were fed to an A-D converter (CED, Sci. Prov., Cambridge) and digitally stored for off-line analysis. Crosscorrelation were analyzed using the Spike2-software package. Phase shifts in cross-correlograms were calculated with respect to the deviation of the first peak from zero.

Study Nr.3

One day after training, retrieval of conditioned fear and associated network activities were assessed in a neutral context. Data analysis was done for periods of 10 s before commencement of acoustic stimuli (pre-stimulus), as well as during CS- and CS+ presentation, as well as for defined freezing episodes of 5-8 s duration during CS- and CS+. To determine amygdalo-hippocampal theta synchronization, cross-correlograms between the amygdala and hippocampus were calculated using low pass (17.5 Hz; transition gap 11.0) filtered waveforms with (2000 bit; 1 s offset) phase shifts aligned (x = 0) to the maximal positive peak.

2.4. Pharmacology.

In study Nr. 4

A guide cannula (length 10 mm diameter 1 mm) were implanted at 1.8 mm AP, 2.7 mm ML (10° angle) and 5.1 mm DV from Bregma under pentobarbital anesthesia (50 mg / kg) and

fixed to the scull with dental cement. One week after surgery, mice were injected with 0.1 nmol NPS, 0.01 nmol NPS or vehicle (saline) at a volume of 0.3 µL per animal. After completion of experiments, cannula location was verified histologically; only correctly implanted animals were considered for statistical analysis (Fig 2.4). NPS was obtained from Phoenix Europe GmbH, Karlsruhe, Germany.

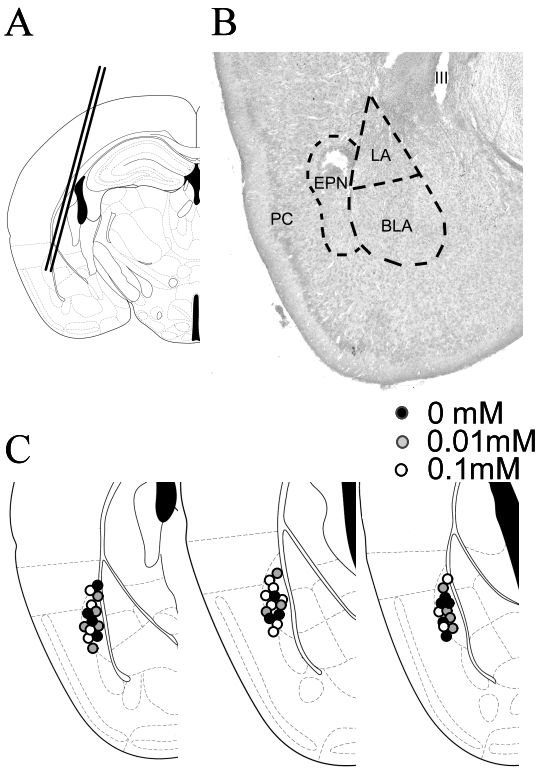


Fig. 2.4. **Probe location in the EPN.** (A) Injection cannula were stereotactically implanted at a 10° angle, aiming at the dorsal portion of the EPN. (B) Histological verification of probe location in a cresyl violet stained coronal section from a representative animal. A small lesion indicates the injection site, which is located in the dorsal EPN. LA, lateral amygdala; BLA, basolateral amygdala; EPN, endopiriform nucleus; PC, piriform cortex; III, third ventricle. (C) Summary of injection sites in individual animals of all three test groups. Illustrations modified from Paxinos and Franklin 2001.

2.5. Data analysis.

In study Nr. 1

Statistical analyses of electrophysiological and behavioral data were done with 1- or 2-way ANOVA with Bonferroni post-hoc test and students t-test, as applicable, by GraphPad Prism 4 program. The data derived from experiments with auditory fear conditioning were assessed by a 2-way ANOVA for repeated measures (CS, time) with CS (CS+, CS-) and time after training (baseline, 2 h, and 24 h) as repeated measured factors. The data derived from experiments with foreground contextual fear conditioning were assessed by a 1-way ANOVA for repeated measures (time) after training (2 min, 30 min, 2 h, and 24 h) as repeated measured factors. Data from background contextual conditioning were analyzed by students t-test.

Study Nr. 2

Statistical analysis of electrophysiological and behavioral data was done with repeated measures (context, CS- and CS+) ANOVA between groups ($NCAM^{-/-}$ vs. $NCAM^{+/+}$), keeping the criterion for significance at $P<0.05$.

Study Nr.3

Statistical analyses of the behavioral data were done with multivariate ANOVA, comparing the 3 genotypes ($GAD65^{+/+}$, $GAD65^{+/-}$ and $GAD65^{-/-}$) over 2 min periods of context exposure, all four CS- including ISIs and all four CS+ including ISIs. Post hoc comparisons were done between genotypes, as well as between tests (STM vs. LTM 24 h, LTM 24 h vs. LTM 14 d, and LTM in the neutral context vs. LTM in the shock context) for each genotype using Fisher's Protected Least Significant Difference (PLSD) test. To control for general changes in fear and anxiety-related behaviors, I determined freezing and risk assessment behavior before (last 2 min of the second adaptation period) and 2 min immediately after training. For

statistical comparison with ANOVA and post hoc (PLSD), cross-correlation maxima in the theta frequency range was determined for each recorded episode and compared between stimuli and animal groups.

In study Nr. 4

Treatment groups (0.1 nmole NPS, 0.01 nmole NPS or vehicle) were compared with one-way analysis of variance (ANOVA) and post-hoc with Fischer's protected least significance difference (PLSD) test.

3. Results and Discussion.

I have investigated the involvement of theta oscillatory activity in amygdala hippocampal pathways during retrieval of learned fear. As a first step, the temporal specificity of the amygdalo-hippocampal theta synchronization was determined (Narayanan *et al*., 2007). Then I began to address the neural mechanisms that underlie such network activity changes in relation to stress and fear memory consolidation. I have employed stress sensitive deficit mutant mouse model in the neural cell adhesion molecule NCAM ($NCAM^{-/-}$) to study whether behavioral deficits in contextual fear memory are reflected in changes of theta synchronization (Albrecht *et al*., submitted). I then undertook a detailed behavioral and electrophysiological investigation of fear memory generalization in GAD65 deficient ($GAD65^{-/-}$) mice (Bergado-Acosta *et al*., 2008). Finally, I tested the hypothesis that the recently discovered neuropeptide S (NPS), which modulates GABAergic transmission in the BLA via the endopiriform cortex is capable to modulate different aspects of fear memory (Meis *et al*., 2008). My data suggest that GABAergic transmission controls amygdalo-hippocampal network activities during a late phase of fear memory consolidation, which is critical for the specificity and stress modulation of fear memory.

3.1. Amygdalo-hippocampal correlate of fear memory.

Paper title: Dissociated theta phase synchronization in amygdalo-hippocampal circuits during various stages of fear memory.

Authors: Narayanan R.T., Seidenbecher T., Kluge C., Bergado J., Stork O., and Pape H-C.

Published in: Eur J Neurosci. 2007. 25: 1823-1831.

Contribution: Experimental design, preparation of the animals, recordings, general discussion.

3.1.1. Background and Rationale.

Amygdala and hippocampus are linked to each other functionally and anatomically (Pitkanen *et al*., 2000) and evidence suggests the involvement of both structures in the formation and retrieval of fear memories (McGaugh *et al*., 1996; LeDoux, 2000). The amygdala is considered as a critical target of plasticity and information storage during cued and contextual fear conditioning, while the hippocampus processes contextual and temporal information of the aversive situation. Auditory and nociceptive information converge on single neurons into the LA of the amygdala whereas contextual information is relayed from the hippocampus mostly into the BLA. Emotional situations induce long-term plasticity in the amygdala and hippocampus suggesting that these regions have a dynamic interrelationship (Diamond *et al*., 2007; Richter-Levin, 2004). Theta oscillations may provide a means for such interactions (Pape *et al*., 2005). Nevertheless, the specific involvement of the amygdala to long-term memory storage and in particular the interplay with the hippocampus is still a matter of debate (Cahill and McGaugh, 1998; Paré *et al*., 2002).

Memory formation is known to involve distinct phases of consolidation, some of which are susceptible to disruption by stress (Diamond, 2006; Tronson and Taylor, 2007). Hence it may

be expected that amygdalo-hippocampal interactions are contributing differentially to specific phases of fear memory consolidation. This study was designed to determine whether the previously observed theta synchronization (Seidenbecher *et al*., 2003) occurs only at specific times following memory acquisition and thus may be linked to particular molecular and cellular processes in the amygdalo-hippocampal system. Therefore wild type mice were implanted with recording electrodes to the CA1 region of the hippocampus and the LA as described previously, and synchronization of theta rhythm oscillations was determined during retrieval sessions performed at (2 min, 30 min, 2 h, or 24 h) after the training. In addition we investigated if the observed increased theta affects expression of fear behaviors and associated theta activity during cued, background as well foreground conditioning.

3.1.2. Results.

Neurophysiological activity.

As a first step, the stimulus associated network activity was determined. Field recordings during the 10s stimulus were used for the calculation of croscorrelograms. For quantitative comparison between different groups, we averaged the respective crosscorrelograms by taking the second positive peak as a quantitative measure (Seidenbecher *et al*., 2003) (Fig. 3.1.1A). By using these second peak values, 2-way ANOVA for repeated measures were performed. The results indicated a time-dependant interaction between groups ($F_{2.28}$=7.26; P<0.0029). Bonferroni post-hoc tests for the cued conditioning revealed that the correlation values during CS+ (y=0.168±0.019) and CS- presentation (y=0.056±0.019) were significantly different (P<0.01) 24 hours after conditioning. No such differences were found between CS- and CS+ presentation at baseline (CS-, y=0.045±0.011; CS+, y=0.02±0.003); and at 2 hours after conditioning (CS-, y=0.0815±0.02; CS+, y=0.08±0.01; Fig. 3.1.1B). Moreover, values during CS+ presentation at 24 hours were significantly different (y=0.168±0.019, $F_{2.14}$ =14.48;

P<0.0004) from those during CS+ presentation at baseline (y=0.02±0.003) and at 2 hours (y=0.08±0.01) as well.

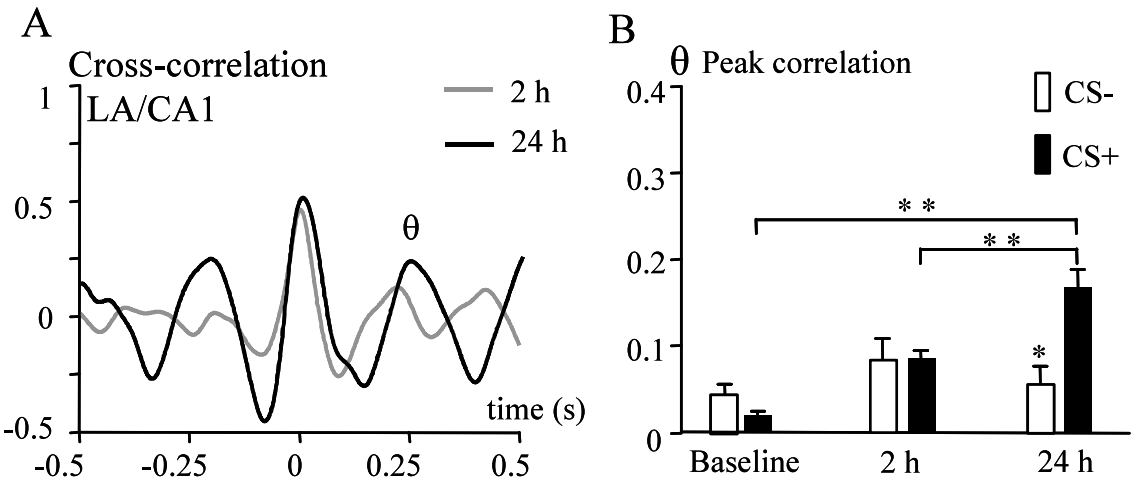


Figure 3.1.1. **Activity in CA1 / LA in cued conditioned animals at various times after conditioning**. (A) Croos correlations at 2 h and 24 h. during CS+ presentation. (B) Mean values of correlation in cued conditioned animals upon CS+ and CS- presentations during baseline, at 2 h, and 24 h. Note significant increase in theta synchronization during CS+ presentation at 24 h post-training.

Freezing behavior

Cued fear conditioned responses were compared before as well as 2 hours and 24 hours after conditioning. Two-way ANOVA for repeated measures with time as factor revealed a significant interaction between the groups ($F_{2,42}$=57.89; P<0.0001). Before conditioning (baseline), animals did not show significant freezing to the acoustic stimuli although low levels of freezing were occasionally observed in response to the novel CS+ (7.00±2.70 % of total time; Fig.3.1.2A). However, pronounced and similar freezing was observed in response to CS+ presentation at 2 hours (62.40±3.60 %) and 24 hours (65.60±3.10 %) post-training (P<0.0001, compared to baseline CS+ freezing). Moreover, during retrieval at both short- (2 h) and long (24 h) term stages, the freezing response to the CS+ was significantly larger than to the CS- (P<0.0001; Fig. 3.1.2A).

Results in the foreground contextual conditioning experiments were similar to those observed in the cued conditioning experiments. Before training, animals did not show conditioned contextual freezing (Fig. 3.1.2B). Following conditioning, exposure to the shock context induced associative freezing responses at 2 minutes, 30 minutes, 2 hours and 24 hours post-training. Freezing duration was similar at all tested stages (2 min, 28.90±3.70 %; 30 min, 20.99±9.20 %; 2 h., 48.00±15.30 %; 24 h, 40.00±16.73 %). One-way ANOVA for repeated measures revealed no difference at the different stages ($F_{3.12}$ =1.2; P=0.3514). As in foreground context conditioning, freezing in the background context could not be detected during the pre-training period (baseline), but was high during retrieval at short-term (2 h, 63.00±7.60 %) and long-term stages (24 h, 55.40±6.60 %) (Fig. 3.1.2C).

Correlation-associated freezing.

In the next step theta phase synchronization was analyzed during episodes (3-4 s) of freezing behavior. Cross-correlograms obtained showed a significant increase in theta phase synchronization (P<0.0001) between the LA and CA1 during retrieval at long-term stages (24 h, y=0.316±0.02) when compared to short-term memory (2 h, y=0.187±0.02) (Fig. 3.1.2). One-way ANOVA for repeated measures of cross-correlations from freezing-associated activity in CA1 / LA at different times after foreground contextual conditioning demonstrated a significant level of interaction over time ($F_{3.12}$=13.91; P=0.0003). Bonferroni post-hoc tests revealed high levels of theta synchronization at 24 hours (y=0.33±0.036), but not at 2 minutes (y= 0.09±0.01; P< 0.001), 30 minutes (y=0.084±0.012; P<0.001), or 2 hours (y= 0.14±0.021; P<0.01) compared to the 24 h memory stage (Fig. 3.1.2B). Background contextual freezing episodes during retrieval 24 hours post-training showed increased theta phase synchronization (y=0.27±0.02) compared to freezing episodes during retrieval at 2 hours after training (y=0.149±0.016; P<0.001).

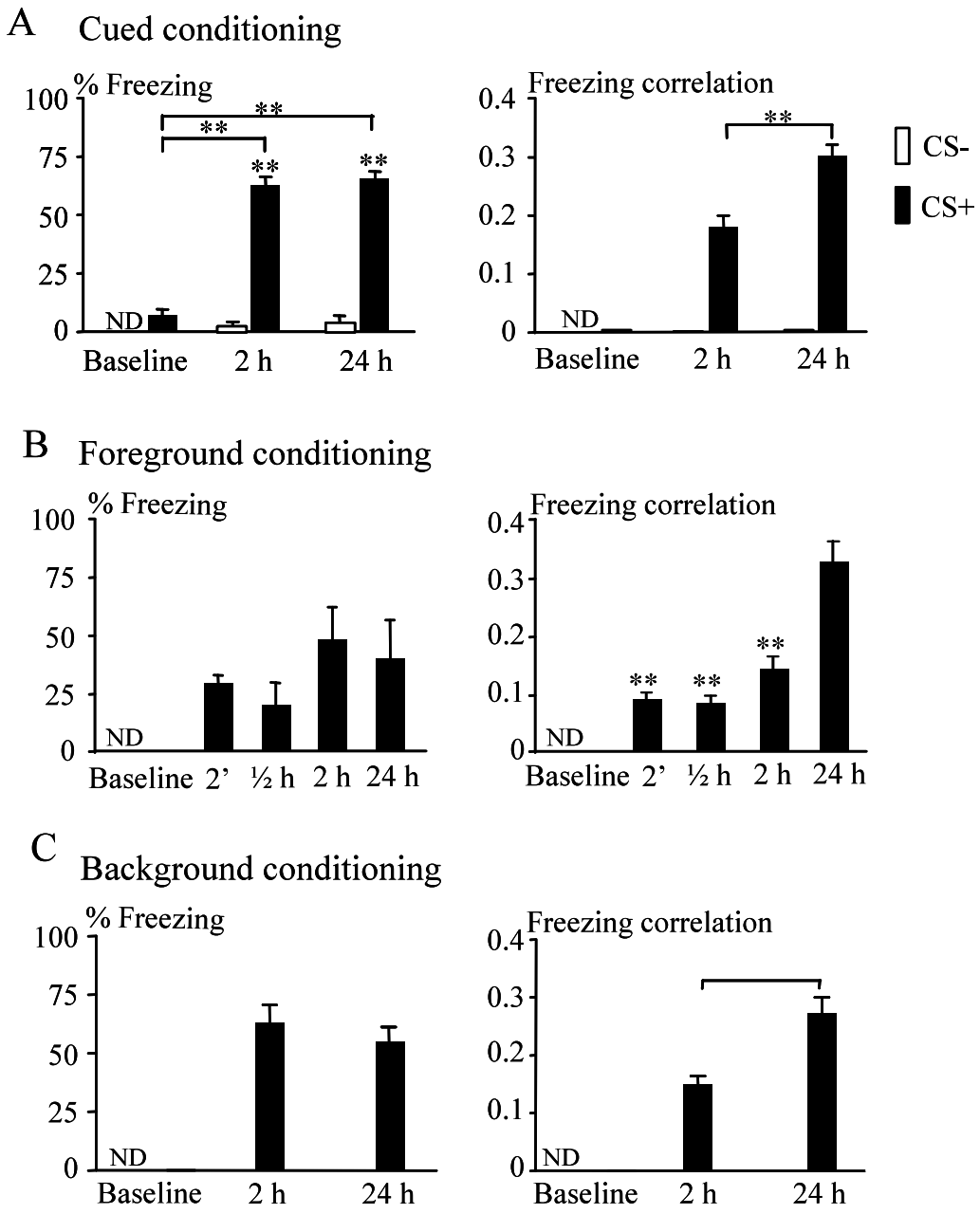


Fig 3.2.1.**Freezing behavior and theta activity associated in CA1 and LA of conditioned animals at various times after conditioning**. (A) Cued conditioning freezing during baseline, and at 2 h and 24 h post-training. Cross-correlation analysis from 3-4 s freezing periods shows a significant increase in freezing-associated theta synchronization at 24 h after training. (B) Foreground conditioning and (C) Background conditioning responses. (Data are means±SEM; *, P<0.05).

3.1.2. Discussion.

This study provides evidence for an involvement of amygdalo-hippocampal theta phase synchronization in the retrieval of cued and contextual informations at long-term (24 hours post training), but not at short-term (2 minutes, 30 minutes, 2 hours) stages of fear memory. This indicates an association with specific cellular and molecular processes of fear memory consolidation and demonstrates that theta synchronization is not involved in the expression of conditioned freezing behavior per se. A similar and temporally 24 h. specific increase in theta synchronization during retrieval of cued as well as background and foreground context fear memory thus supports the general relevance of this observation.

The importance of amygdala plasticity for the acquisition of fear memory is widely accepted, but still is not know whether the amygdala is itself the locus of fear memory storage (Paré, 2002) or is involved in the modulation of memory in other brain areas. An interaction with the hippocampus is required for the integration of contextual and temporal information during memory formation (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Maren and Fanselow, 1997). Explicit cues, defined through their appearance for a short period and specific physical characteristics (Grillon and Davis, 1997) generally are more salient than contextual ones (Rescorla, 1976), but the latter can facilitate the retention of cued fear memory (Philipps and LeDoux, 1992). Thus, information from the hippocampal formation, reaching at the BLA (Canteras and Swanson, 1992), can strongly impede on emotional stimulus associations. Whereas lesions of the amygdala abolish cued conditioning, a hippocampal lesion will abolish conditioning of the freezing response to the conditioning chamber (Phillips and LeDoux, 1992).

In summary, the increased theta correlation between LA / CA1 only reflect a transient association of fear memory, adding to the previous debate about the assumed function of amygdala as a place of fear memory storage (Cahill and McGaugh, 1998; LeDoux, 2000; Paré *et al*., 2002). This study does not exclude regional or functional specificity, however the

results indicate that the amygdalo-hippocampal network is a dynamic system that could change ensemble activities in different brain regions. Theta oscillatory activities in this pathway only after 24 h may represent memory reactivation of a previous consolidation process that stabilizes the memory in this circuit. Theta moreover maybe induce expression of new genes, thought to be involves in the consolidation of fear memories (Stork *et al*., 2001).

3.2. NCAM in amygdalo-hippocampal interactions.

Paper title: Role of NCAM in amygdalo-hippocampal interactions and stress modulation of contextual fear memory.

Authors: Albrecht A,. Bergado-Acosta J.R,. Pape H-C., and Stork O.

Submitted to: The International Journal of Neuropsychopharmacology.

Contribution: Design, conduction, statistical analysis and interpretation of the electrophysiological experiments.

3.2.1. Background and Rationale.

In the first study we proposed that theta rhythms could drive gene expression and remodeling of the synaptic matrix that is thought to occurs during memory formation (Sjöström *et al*., 2008). One possible candidate for such function is the neural cell adhesion molecule (NCAM) (Fields and Itoh, 1996). NCAM have been implicated in the reorganization of synaptic connections, as well as learning and memory consolidation (Welzl and Stork, 2003; Hartz and Rønn, 2008). The expression of the protein in the hippocampus seems to be regulated depending on stimulus intensity, in a time depended manner across different cell populations (Lopez-Fernandez *et al*., 2007; Merino *et al*., 2000). In the amygdala NCAM-PSA dissociation was reported to increase fear memory extinction (Markram *et al*., 2007a). Behavioral analysis of null NCAM mutant mice showed several disturbances in cue and contextual fear memory, with changes in aggressive, anxiety and antidepressant-like behavior (Stork *et al*., 1999; 2000).

These results were extended by us (Albrecht *et al*., *in revision*) showing that the deficit of $NCAM^{-/-}$ mice in contextual fear memory could be overcome by pre-adaptation of animals to the conditioning box. Moreover, a lower level of contextual freezing behavior was evident in $NCAM^{-/-}$ mice compared to their $NCAM^{+/+}$ littermates after overtraining. We also provide

evidence for an increase of NCAM mRNA in the LA upon foreground context conditioning, 6 hour after training, and a significant reduction of expression in the BLA following foreground contextual conditioning and cued overtraining. These data together suggest a role of NCAM in stress-modulation of contextual fear memory and a contribution of the amygdala to this function. Therefore, I was tented to investigate the potential consequences of the NCAM null mutation on amygdalo-hippocampal theta synchronization as described in study Nr. 1. My goal was to determine the possible role of NCAM in the generation of theta synchronization between amygdala and hippocampus in over-trained $NCAM^{-/-}$ mice and $NCAM^{+/+}$ littermates during contextual and cued retrieval memory. To this end, NCAM littermates after 2 adaptations (6x CS-) and 2 overtraining sessions (10 CS+ / US pairings at 0.6 mA foot shock) were re-exposed to the training context 24 h later as well as CS- and CS+. During the retrieval session, in addition to LA / CA1 field potentials, freezing and risk-assessment like-behaviors were recorded.

3.2.2. Results.

Behavior analysis of implanted animals corroborated the reduction of freezing behavior of $NCAM^{-/-}$ mice during context presentation (mean±S.D.; 8.2±13.1 vs. 46.3±46.5 for $NCAM^{+/+}$). Overtraining protocols induced a strong generalization of the freezing response to the CS- that was most pronounced in the wild type mice (mean±S.D.; 39.5±27.6 vs. 23.9±18.1 for $NCAM^{-/-}$). The freezing response to the cue was very high and similar for both, $NCAM^{+/+}$ (mean±S.D 60.2±19.2 vs 66.2±23.8for $NCAM^{-/-}$). Theta synchronization between amygdala and hippocampus was reduced in $NCAM^{-/-}$ mice throughout the retrieval session (Fig 3.2.1). One way-ANOVA showed a general significant group effect in theta-synchronization in $NCAM^{-/-}$ mice during context re-exposure ($F_{1.10}$=8.259; P=0.021), during the CS- ($F_{1.10}$=18.014; P=0.003) and CS+ ($F_{1.10}$=8.814; P=0.018) (mean±S.D; Context, 0.037±0.022

vs. 0.200±0.043 for NCAM$^{+/+}$; CS-, 0.009±0.009 vs. 0.209±0.037 for NCAM$^{+/+}$; and CS+, 0.093±0.038 vs. 0.2378±0.031 for NCAM$^{+/+}$).

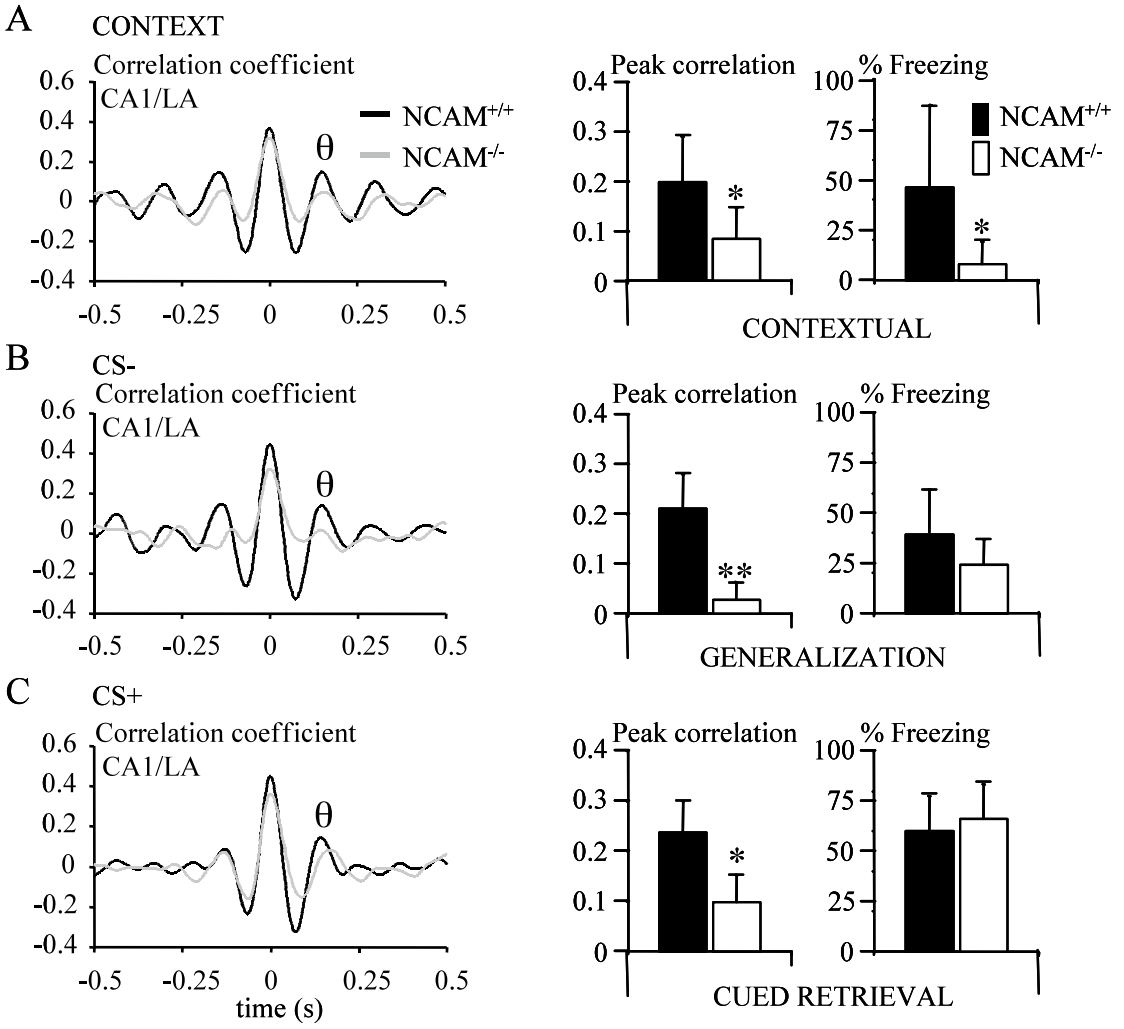


Figure 3.2.1. **Reduced amygdalo-hippocampal theta synchronization in NCAM$^{-/-}$ mice**. Averaged cross correlograms of theta activity in the basolateral complex and in the CA1 region of hippocampus in different phases of auditory cued memory retrieval. Note the reduced peak synchronization in the theta frequency range (θ) in overtrained NCAM$^{-/-}$ mice in all stages of fear memory retrieval (A,B and C), accompanied by a reduced fear response towards the background conditioning context but normal responses to the CS- and CS+. Values are mean±SEM. *, significant differences between NCAM$^{-/-}$ and NCAM$^{+/+}$ mice, P<0.05.

3.2.3. Discussion.

In this work I have addressed neural activity patterns in the amygdalo-hippocampal pathway of fear conditioned NCAM null mutant mice. Strikingly, overtrained NCAM$^{-/-}$ mice showed low amygdalo-hippocampal theta synchronization during exposure to context, CS- and CS+ presentation and reduction of freezing during contextual retrieval. NCAMs have emerged as cell-cell mediators of long-lasting synaptic changes during learning and memory formation (Senkov *et al*., 2006; Benson *et al*., 2000; Bonfanti, 2006), they also activated cellular signaling that control short- and long-lasting structural membrane associations (Aonurm-Helm *et al*., 2008; Diviani and Scott, 2001). The changes in NCAM expression have been shown to be time, stress and region dependent (Sandi 2004; Sandi *et al*., 2005), indicating that NCAM is expressed in specific phases during memory consolidation, being down-regulated at first in order to allow re-organization of synaptic connections (Hildebrandt *et al*., 2007).

The regulation of these processes through NCAM seems to be strongly influenced by polysialic acid (PSA), a large carbohydrate polymer (Bonfanti, 2006; Hartz and Rønn, 2008). PSA may play a permissive role in plasticity by decreasing homophilic adhesion of NCAM in that way facilitating neurite outgrowth and synaptic adjustment (Hildebrandt *et al*., 2007). PSA-NCAM expression and function on the other hand are closely related to stress levels and glucocorticoid activity (Sandi, 2004). For example, their expression in the hippocampus after contextual fear training differs with different stressor intensities (0.2, 0.4, and 1.0 mA) or time factors (12 h or 24 h) (Merino *et al*., 2000; Sandi *et al*., 2003). Region-specific changes in NCAM expression are also seen depending on the training paradigm. For example, PSA-NCAM upregulation is observed only in the dorsal but not ventral DG at 24 hours only for contextual but not for cued fear conditioning. Specific removal of PSA through microinfusion of the enzyme endoneuraminidase-N in the dorsal hippocampus reduced freezing responses only to the conditioned context (Lopez-Fernandez *et al*., 2007). Two enzymes, ST8SiaII and ST8SiaIV, mediate polysialylation of the NCAM core protein. Mice lacking the

polysialyltransferase ST8SialV/PST (Markram *et al*., 2007a), responsible for attachment of PSA to NCAM in adulthood, showed a mild deficit only in hippocampal contextual learning. In the amygdala cleavage of PSA-NCAM did not affect acquisition, consolidation or expression of remote fear memories. However, intra-amygdaloid micro-infusions of the enzyme endoneuraminidase-N enhanced fear extinction processes (Markram *et al*., 2007b). Sugessting that NCAM function in the amygdala may largely be determined by changes in the core protein rather than its polysialation.

NCAM molecules are also concerned in cell interactions during nervous system development (Rønn *et al*., 1998), thus I cannot rule out an involvement of developmental deficits onto my observations. Postnatal inactivation of NCAM in the hippocampus has been achieved recently by means of CRE-loxP recombination using the CaMKII promoter, and provided a phenotype with reduction of LTP in CA1 and reduced precision of spatial learning for the water maze (Bukalo *et al*., 2004). Since the CamKII promoter is also active in the amygdala, an analysis of such conditional mutant mice would therefore be a very valid alternative approach in future studies. In line with the observed behavioral deficits during pre-adaptation, theta synchronization between LA-CA1 was not observed in the $NCAM^{-/-}$ mice after 24 h, neither in context, generalization or cued retrieval. Our data thus indicate that theta, once more, is not required to express freezing but may be involve in controlling cellular plasticity.

The interplay of stress and memory formation has been long discussed, it is assumed that high emotional events could enhance performance under easy learning conditions, but impairs performance when the learning situation is challenging or stressful to a large extent (Hanoch and Vitouch, 2004; Diamond *et al*., 2007). Thus, an involvement of NCAM in learning and LTP (Cremer *et al*., 1998) has been supported by the analysis of mice deficient in NCAM as outlined above (Bisaz *et al*., 2009). $NCAM^{-/-}$ mutants can be seen as a model of stress hypersensitivity, they show disturbed contextual and cued memories, changes in aggressive, anxiety and antidepressant-like behavior and hypersensitivity to the anxiolytic serotonin1A

(5-HT1A) receptor agonist buspirone (Stork *et al*., 1997; 1999; 2000). In fact similar changes are observed also in mutant mice with disturbance of serotonergic function, for example in mice carrying targeted disruption of the genes 5-HT1A receptor function (Bert *et al*., 2008). Finally deficit in contextual fear conditioning are observed in NCAM transgenic mice expressing a soluble fragment of NCAM consisting of its extracellular domain (Pillai-Nair *et al*., 2005). These deficits were associated with impairment in GABAergic synaptic connectivity (Brennaman and Maness, 2008) indicating the involvement of GABAergic processes in the NCAM-deficiency induced deficit of salience coding.

3.3. Role of GAD65 in fear memory consolidation.

Paper title: Critical role of the 65kD isoform of glutamic acid decarboxylase in consolidation and generalization of Pavlovian fear memory

Authors: Bergado-Acosta J.R., Sangha S., Narayanan R.T., Obata K., Pape H-C., and Stork O.

Published in: Learning & Memory. 2008. 15: 163-171.

Contribution: Design, conduction, statistical analysis and interpretation of the behavioral and electrophysiological experiments.

3.3.1. Background and Rationale.

GABAergic interneurons play a key role in determining neural excitability and information processing in the amygdala and hippocampus (Pape and Stork, 2003, Kullmann and Lamsa, 2007). Further evidence suggests that GABA is modulated by stress, and changes in the expression of GABAergic factors have recently been shown following fear conditioning training (Rodriguez-Manzanares *et al*., 2005; Isoardi *et al*., 2004). Indeed, transient changes of gephyrin expression and $GABA_A$ receptor binding are evident in the amygdala 2-6 h after fear conditioning (Chhatwal *et al*., 2005). Concerning the hippocampus, it has been shown that deficits in $GABA_A$ receptor clustering increase anxiety in mutant mice heterozygous for the γ2 subunit of the $GABA_A$ receptor (Crestani *et al*., 1999) and that post-training injections of the benzodiazepine midazolam interferes with context memory consolidation (Gafford *et al*., 2005). Recently it has been suggested that two fundamentally different components exist in fear memory, a stimulus-specific memory and a non-associative sensitization (Siegmund and Wotjak, 2007). The interplay of these components may determine strength, persistence, and specificity of the memory. Changes in GABAergic inhibition in the amygdala and hippocampus are likely to contribute to this balance.

We have previously reported specific regulation of the GAD65 isoform in the basolateral complex of the amygdala, 24 h after fear conditioning training (Pape and Stork, 2003; Bergado-Acosta *et al*., 2008). GAD is expressed in the majority of GABAergic neurons in the amygdala and the hippocampus, and modulation of GAD65 and GAD67 expression by acute and chronic stress has been earlier reported (Bowers *et al*., 1998). In particular, GAD65 is considered to be the activity-dependent and synaptically localized isoform providing GABA for phasic inhibition, whereas GAD67, as the cytosolic isoform, is critical for metabolic GABA synthesis and tonic inhibition. The reduced GAD65 expression may be taken as an indicator of transiently reduced GABAergic neuronal activity during fear memory consolidation or retention. In fact, different forms of synaptic plasticity have been demonstrated in GABAergic neurons of the LA / BLA and hippocampus using electrophysiological tools (Mahanty and Sah, 1998; Bauer and LeDoux, 2004; Kullmann and Lamsa, 2007). On the other hand, the transient GAD gene regulation itself may result in an altered inhibition within the lateral and basolateral amygdala nuclei as well as the hippocampus (Shimizu *et al*., 2005; Szinyei *et al*., 2007). Therefore, in this study I aimed to investigate the particular role of GAD65 in fear memory consolidation. To this end I determined behavioral responses of GAD65 null mutant mice during retrieval at different time points. In addition, I investigated synchronization of theta oscillation in amygdala and hippocampus, in the manner established in studies 1 and 2.

3.3.2. Results.

Behavior of GAD65 mutant mice.

I investigated, in a differential fear conditioning paradigm, memory formation and its specificity in GAD65 mutants. Two acoustic stimuli (CS- and CS+) were employed and memory retrieval was tested at different time points after training (30 min, 24 h, and 14 d) and

in different (neutral and training) contexts. To minimize a potential role of the CS- as an explicit safety signal in our differential conditioning paradigm, I presented this test stimulus in conjunction with the training context repetitively prior to but not during the training session. We have previously employed this procedure to demonstrate overtraining-induced generalization of fear memory and observed that inhibitory conditioning to the CS- is only observed when the CS+ itself is presented in explicitly unpaired fashion (Laxmi *et al*., 2003).

Retrieval respose in the neutral context.

Testing the specificity of long-term fear memory in GAD65 mutant mice, I observed a significant genotype x stimulus interaction for freezing behavior ($F_{2.61}$=13.806; P<0.0001). My results confirm previous observations (Stork *et al*., 2003) of reduced freezing behavior of GAD65$^{-/-}$ mice during the CS+ test period (GAD65$^{+/+}$ 28.5±13.2 %; GAD65$^{+/-}$ 23.8±13.2 %; and GAD65$^{-/-}$ 11.3±5.3 % all values are mean±S.D, $F_{2.31}$=6.274; P<0.006) (Fig. 3.3.1A). Risk-assessment behavior in contrast was not different between groups (Fig. 3.3.1B). Reduced freezing of GAD65$^{-/-}$ mice was evident specifically during CS+ tone presentations (repeated measures ANOVA, genotype effects: $F_{2.29}$=12.472; P<0.0001) and not significant during ISIs. All groups reduced their freezing response gradually upon repetitive stimulation (within-subject comparison, $F_{3.87}$=6.509; P<0.001) (Fig. 3.3.1D).

Strikingly, GAD65$^{-/-}$ mice also showed a significantly increased freezing response during the CS- test period (8.8±6.2 %), in contrast to their GAD65$^{+/+}$ (0.1±0.2 %), or GAD65$^{+/-}$ (0.2±0.2 %) littermates (P<0.0001). Thus, while wild types and heterozygots both clearly differentiated between the acoustic stimuli (P<0.0001), homozygous mutants displayed similar levels of freezing during both CS+ and CS- test periods (P>0.24). Similar to freezing, risk-assessment behavior (Fig. 3.3.1B) was significantly affected by genotype during the CS- test period ($F_{2.31}$=29.025; P<0.0001), showing a selective increase in GAD65$^{-/-}$ mice as compared to their GAD65$^{+/+}$ (P<0.0001) and GAD65$^{+/-}$ (P<0.0001) littermates. Further analysis revealed that generalized freezing behavior of GAD65$^{-/-}$ mice was specific to the CS- tone presentation and

not seen in the ISIs (Fig. 3.3.1C). Repeated measures ANOVA showed a significant effect of genotype across the CS- presentations ($F_{2.29}$=23.150; P<0.0001) and a significant stimulus x genotype interaction ($F_{6.87}$=6.538; P<0.0001), due to efficient habituation of the generalized response in GAD65$^{-/-}$ mice (within-subject comparison: $F_{3.33}$ = 8.537 P<0.0001). Post hoc analysis confirmed increased freezing behavior of GAD65$^{-/-}$ mice in response to the first and second (P<0.001) as well as the third CS- tone (P<0.05), when compared to GAD65$^{+/+}$ mice. No such increase was observed in GAD65$^{+/-}$ mice.

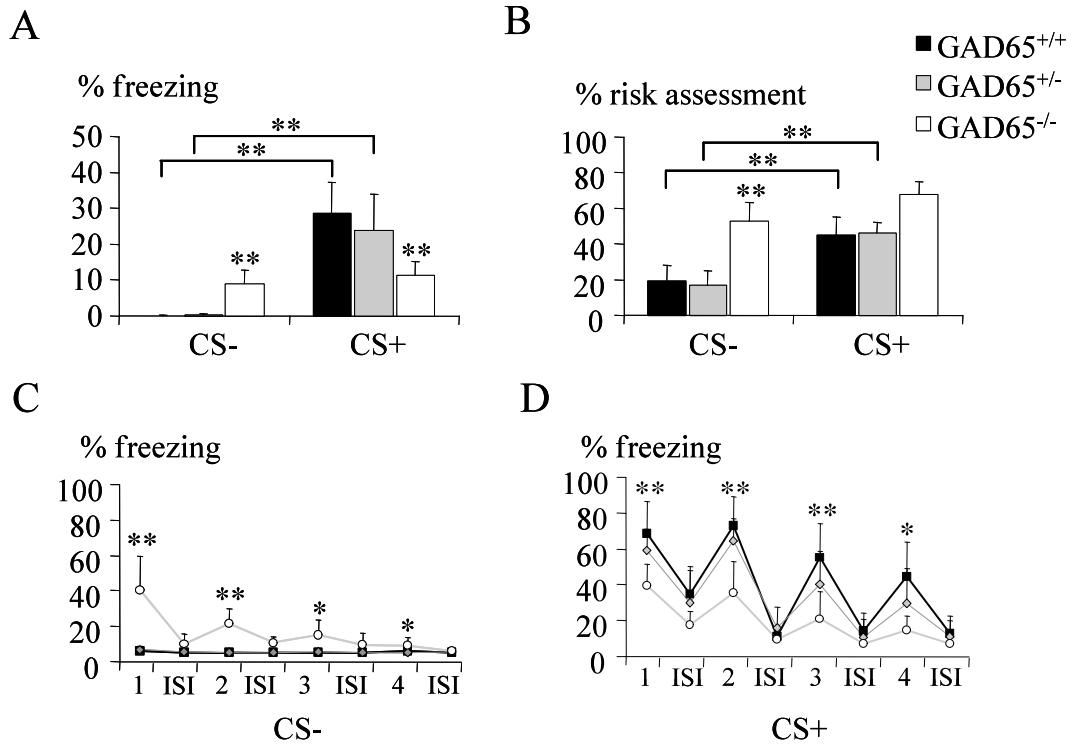


Figure 3.3.1. **Generalization of long-term fear memory in GAD65$^{-/-}$ mice**. (A) GAD65$^{-/-}$ mice displayed increased freezing and (B) elevated risk-assessment behavior towards the CS- during retrieval test 24 h after training. (C) Generalized freezing in GAD65$^{-/-}$ mice was specifically associated with tone presentation during the CS- session, but not seen during inter-stimulus intervals (ISI). (D) Similarly, reduced freezing of GAD65$^{-/-}$ mice to the CS+ was only observed during CS+ tone presentation**.** ** p<0.01, * p<0.05, when compared to GAD65$^{+/+}$. 1-4, stimulus number. Values are mean±SEM.

Sensory and baseline behavior controls.

Control measures of fear-related behavior before (freezing, and risk assessment undetectable) and immediately after fear conditioning (freezing: 10.1-14.9 %, P>0.09; risk-assessment 56.5-61.7 %) did not show any difference between genotypes. Moreover, no indication was found for an altered pain sensitivity in GAD65$^{-/-}$ mice, as genotypes did not differ in their behavioral response to increasing foot shock currents, (first immobilization / vocalization at 0.14 / 0.2 mA in GAD65$^{+/+}$ (n=7), 0.12 / 0.3 mA in GAD65$^{+/-}$ (n=5), and at 0.11 / 0.29 mA in GAD65$^{-/-}$ mice (n=5), respectively).

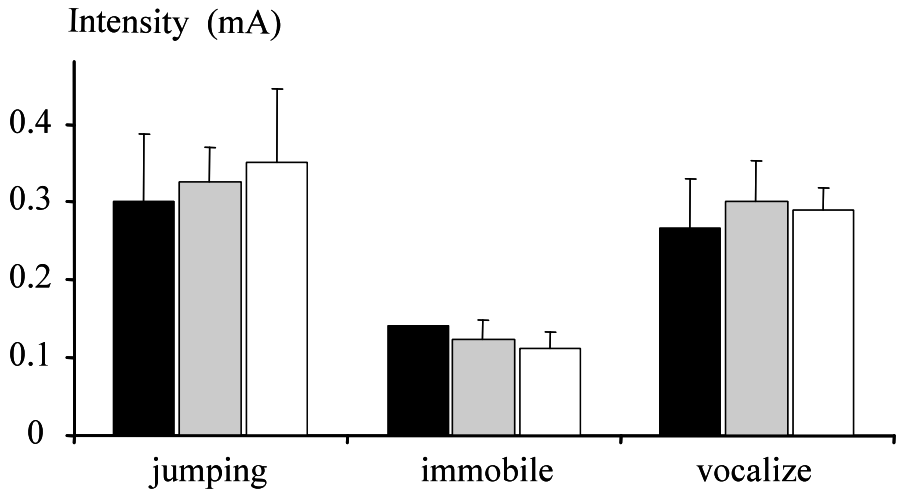


Figure 2.5. **Foot shock sensitivity in GAD65 mice to the fear conditioning apparatus**. No altered pain sensitivity in GAD65$^{-/-}$ mice, as genotypes did not differ in their behavioral response to increasing foot shock currents. Data presented as mean±SEM.

Memory time course.

To investigate whether the observed deficits of GAD65$^{-/-}$ mice may be related to a function of GAD65 in fear memory consolidation, I next analyzed the mutants' performance at different time points after training. Considering the observed changes of GAD65 gene expression in wild types, I decided to investigate fear memory 30 min and 14 d after conditioning. Firstly, during short-term memory retrieval 30 min after training, no change in performance was observed in GAD65$^{-/-}$ mice that would be comparable to their long-term memory deficit. Significant genotype effects could neither be observed during the CS- test period (ANOVA, $F_{2.23}$=0.59; P>0.6 for risk-assessment; P>0.3 for freezing) nor the CS+ test period (P>0.6 and P>0.7, respectively). GAD65$^{-/-}$ mice did not show reduced freezing during the CS+ test period (16.9±9.6 %) or increased freezing during the CS- test period (2.1±2.7 %), when compared to their littermates (Fig. 3.3.2A,B). Comparison between long-term (LTM) and short-term memory (STM) tests showed that GAD65$^{-/-}$ mice failed to increase their freezing response during the CS+ test period upon fear memory consolidation, as opposed to GAD65$^{+/+}$ mice (P<0.015) (Fig. 3.3.1; 2A). Risk-assessment in contrast was different ($F_{1.54}$=13,671 P<0.001) between STM and LTM tests in general; genotype also generally affected behavior performance ($F_{2.54}$=7.895 P<0.001). The differences resulted from the increased risk assessment behavior in GAD65$^{+/+}$ and GAD65$^{+/-}$ mice during STM as compared to LTM testing (Fig 3.3.1;2B). Moreover, a significant test x genotype interaction was found for freezing behavior during the CS- test period ($F_{2.54}$=6.634 P>0.003), due to the selective increase of freezing by GAD65$^{-/-}$ mice in long-term memory retrieval (P<0.001). The comparison of STM and LTM showed analogous differences for risk assessment ($F_{1.54}$=9.348 P<0.004), as the generalization of cued fear memory was not observed in GAD65$^{-/-}$ during STM retrieval.

Secondly, a group of animals was tested 14 d after fear conditioning training (Fig. 3.3.2C,D). At this time point, I observed a stimulus x genotype interaction for both freezing ($F_{2.45}$=9.468;

P<0.0001) and risk assessment behavior ($F_{2.45}$=9.298; P<0.0001) that was similar to the effects in the 24 h LTM group. Freezing to the CS+ was dependent on genotype ($F_{2.22}$=7.728; P<0.003), with GAD65$^{-/-}$ mice showing reduced levels (7.1±9.8 %) compared to both GAD65$^{+/+}$ (26.8±5.0 %; P<0.001) and GAD65$^{+/-}$ mice (20.2±6.9 %; P<0.032). Risk-assessment behavior (25.4-42.9 %) appeared to be somewhat lower in the mutants, yet the difference between genotypes failed to reach significance. During the CS-, a significant effect of genotype was not observed towards freezing behavior, although GAD65$^{-/-}$ mice (7.3±7.3 %) showed a somewhat increased response. However, risk-assessment proved to be dependent on genotype ($F_{2.22}$=7.950; P<0.003), due to an increase in GAD65$^{-/-}$ mice (23.8±8.1 %), compared to GAD65$^{+/+}$ (10.2±8.1 %; P<0.012) and GAD65$^{+/-}$ mice (2.3±1.9 %; P<0.001). Comparing responses within each genotype, it became evident that both GAD65$^{+/+}$ and GAD65$^{+/-}$ mice distinguished well between CS+ and CS- (P<0.001 for both freezing and risk-assessment), whereas GAD65$^{-/-}$ mice did not. Finally, when comparing the different stages (24 h and 14 d) of long-term memory, we found no interaction of test and genotype concerning freezing behavior (CS-: $F_{1.53}$=0.380; P>0.686 or CS+: $F_{1.53}$=0.070; P>0.932), but an interaction was evident for risk-assessment behavior during both CS- ($F_{1.53}$=3.588; P<0.035) and CS+ ($F_{1.53}$=15,237; P<0.0001), which is explained in the reduction of this behavior in the GAD65$^{-/-}$ after 14 days (CS-: P<0.0001 or CS+: P<0.0001) compared to the 24 h time point.

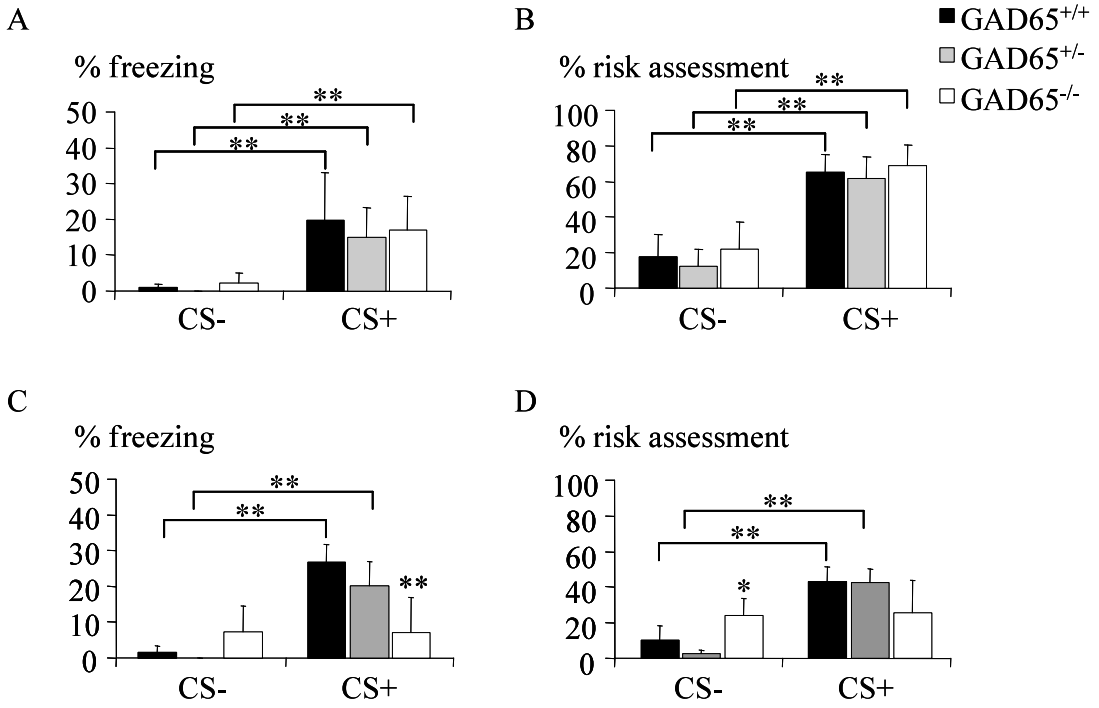


Figure 3.3.2. **Memory time course** (A) Short-term memory retrieval 30 min after training produced specific freezing response towards the CS+ in all groups without evidence for generalization to the CS- in GAD65$^{-/-}$ mice. (B) Similarly, no difference between genotypes was observed in their risk-assessment behavior during the analysis of short-term fear memory. (C) Long-term memory retrieval after 14 d produced a similar result as 24 h LTM, with a reduced freezing of GAD65$^{-/-}$ mice to the CS+ and a failure to discriminate the CS-. (D) Risk-assessment behavior was increased in GAD65$^{-/-}$ mice during 14 d LTM, when compared to their wild type and heterozygous littermates. **, $p<0.01$ and *, $p<0.05$, when compared to GAD65+/+.Values are mean ± SEM.

Retrieval responses in the training context.

As next step, 24 h long-term fear memory was tested in the training context to address the potential importance of contextual background for generalization of auditory cued fear memory in GAD65 mutant mice (Fig. 3.3.3A,B). Background context exposure indeed induced a moderate fear response (freezing: 3.1-6.4 % and risk-assessment: 27.7-39.4 %) in

all genotypes compared to the neutral context ($P<0.0001$). No significant difference of background contextual conditioning was observed between the genotypes ($P>0.3$). However, upon confrontation with the CS+ in the conditioning context, a genotype effect ($F_{2.23}=7.447$; $P<0.004$) similar to that seen in the neutral context emerged; i.e. $GAD65^{-/-}$ mice showed reduced freezing (Fig. 3.3.3C), compared to $GAD65^{+/+}$ ($P<0.001$) and $GAD65^{+/-}$ mice ($P<0.017$). The statistical analysis of context effects (neutral context vs. shock context) revealed a significant genotype x test interaction during the CS- test period (ANOVA $F_{2.54}=7.662$; $P<0.001$ for freezing and $F_{2.54}=16.689$; $P<0.0001$ risk-assessment). While both $GAD65^{+/+}$ and $GAD65^{+/-}$ mice showed a context-dependent increase of freezing and risk assessment during the CS- test period in the shock context ($P<0.003$), the behavioral response of $GAD65^{-/-}$ was similar in both tests (freezing: 8.8±6.2 % neutral context and 4.6±3.1 % shock context; risk assessment: 52.5±13.4 % and 35.7±13.8 %, respectively). Stimulus-by-stimulus analysis, again, revealed a reduced freezing of $GAD65^{-/-}$ mice during CS+ tone presentation, similar to that observed in the neutral context (for comparison see Fig. 3.3.3D). A significant genotype effect ($F_{2.29}=5.384$; $P<0.01$) was observed, with reduced freezing of $GAD65^{-/-}$ mice to the second ($P<0.001$), third and fourth ($P<0.05$) presentations of the CS+ compared to $GAD65^{+/+}$ mice. Again, no significant change was observed in $GAD65^{+/-}$ mice.

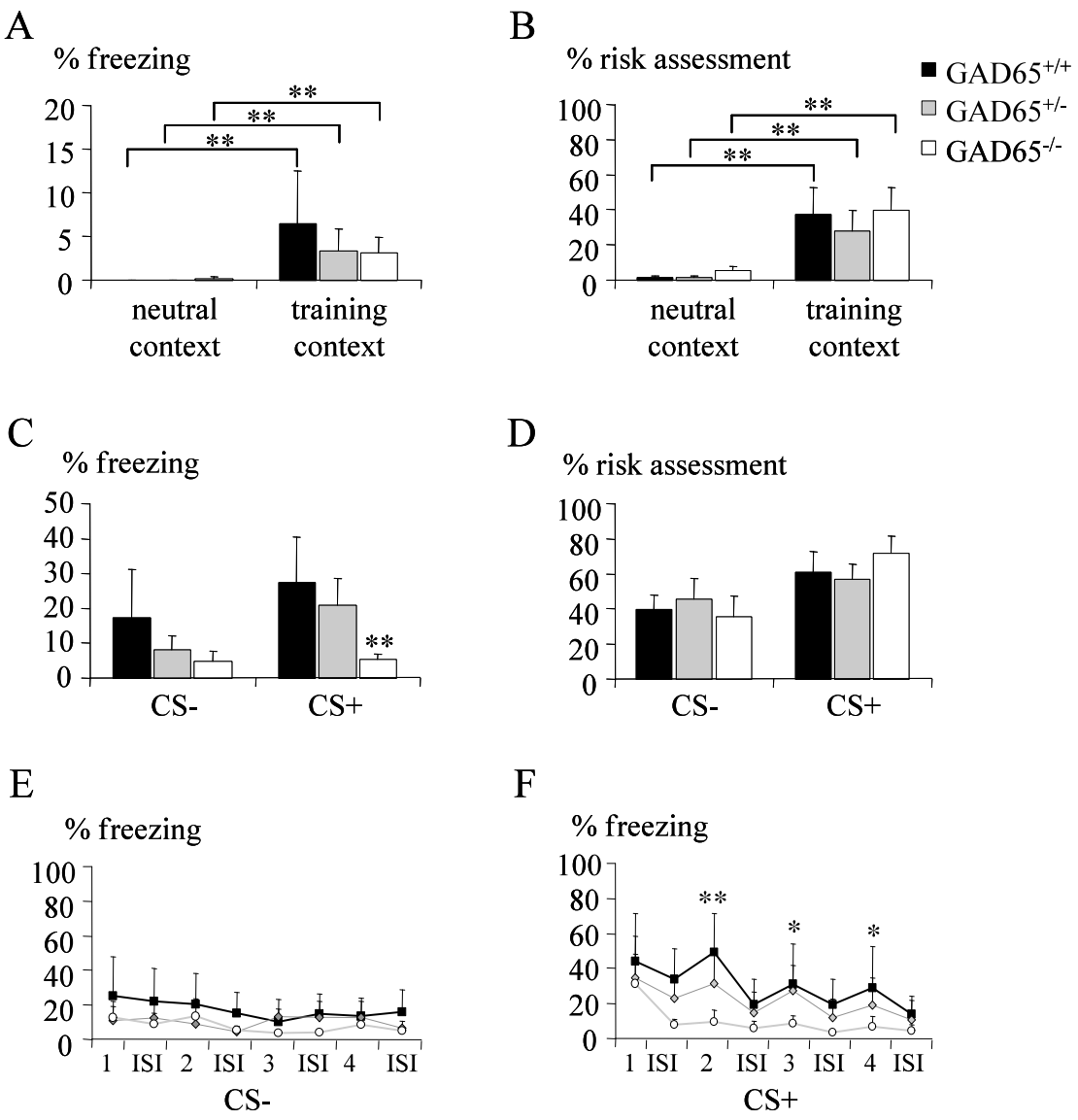


Figure 3.3.3 **Contextual fear memory in GAD65 mice.** (A) During retrieval of background contextual long-term fear memory, freezing and (B) risk assessment responses did not differ between genotypes. (C) GAD65$^{+/+}$ and GAD65$^{+/-}$ mice showed significant freezing to both CS- and CS+ when presented in the training context, GAD65$^{-/-}$ mice displayed low levels of freezing to the CS- and to the CS+. This indicates that the generalization of fear memory in GAD65$^{-/-}$ mice occurred independently of the retrieval context. (D) Risk assessment behavior was not different between genotypes. (E) Detailed analysis of freezing responses across CS- stimuli and ISIs. (F) The freezing response of GAD65$^{-/-}$ mice was specifically reduced during CS+ tone presentation. ** $p<0.01$, * $p<0.05$, when compared to GAD65$^{+/+}$. 1-4, stimulus number. Values are mean±SEM.

Network activity during fear memory retrieval.

To begin to investigate network activity related to fear memory generalization, I next studied neural activities in the lateral amygdala and CA1 region of the hippocampus of fear conditioned GAD65 mutants (representative original recordings shown in Fig. 3.3.4A). By calculating the cross-correlation of activities between these structures, amygdalo-hippocampal theta synchronization was determined before and during exposure to CS- and CS+ stimuli, and during defined freezing episodes (Seidenbecher *et al*., 2003). I also included a group of overtrained GAD65$^{+/+}$ mice to this analysis in order to compare the network activity patterns between genotypes during fear memory generalization. All groups displayed theta synchronization with a peak between 5.9 and 6.5 Hz (i.e. type2 theta) during freezing, as described previously (Seidenbecher *et al*., 2003; Narayanan *et al*., 2007; Fig. 3.3.4B).

Behavioral analysis of the recorded animals confirmed my findings of reduced freezing to the CS+ tone presentation (31.9±27.5 % in GAD65$^{-/-}$ vs. 60.0±13.5 % in GAD65$^{+/+}$, $F_{1.15}$=5.259; P<0.038) and increased generalization to the CS- tone presentation (21.5±16.8 % GAD65$^{-/-}$ vs. 3.3±4.8 % GAD65$^{+/+}$, $F_{1,15}$=5.419; P<0.037) in GAD65$^{-/-}$ mice when compared to their GAD65$^{+/+}$ littermates (Fig. 3.3.5A). Overtrained GAD65$^{+/+}$ mice also showed significant generalization to the CS- (39.5±27.6 %, $F_{1.11}$=8.21; P<0.017 compared to GAD6$^{+/+}$ with standard training) as well as pronounced freezing to the CS+ tone presentation (60.3±19.2 %). When comparing these three groups of mice, we observed significant effects on amygdalo-hippocampal synchronization during both CS- ($F_{2.21}$=7.699; P<0.004) and CS+ tone presentations ($F_{2.21}$=16.396 P<0.0001). During CS-, overtrained animals showed a significant increase in correlation (0.21±0.07), compared with GAD65$^{+/+}$ (0.10±0.06 P<0.019) and GAD65$^{-/-}$ mice (0.06±0.04 P<0.001). This increased synchronization was comparable to the response of both overtrained (0.24±0.05) and standard protocol trained GAD65$^{+/+}$ mice (0.22±0.05) to the CS+. In contrast, GAD65$^{-/-}$ mutant mice failed to increase amygdalo-

hippocampal theta correlation during the CS+ tone presentation (0.05±0.05; P<0.0001 compared to both other groups; Fig. 3.3.4B).

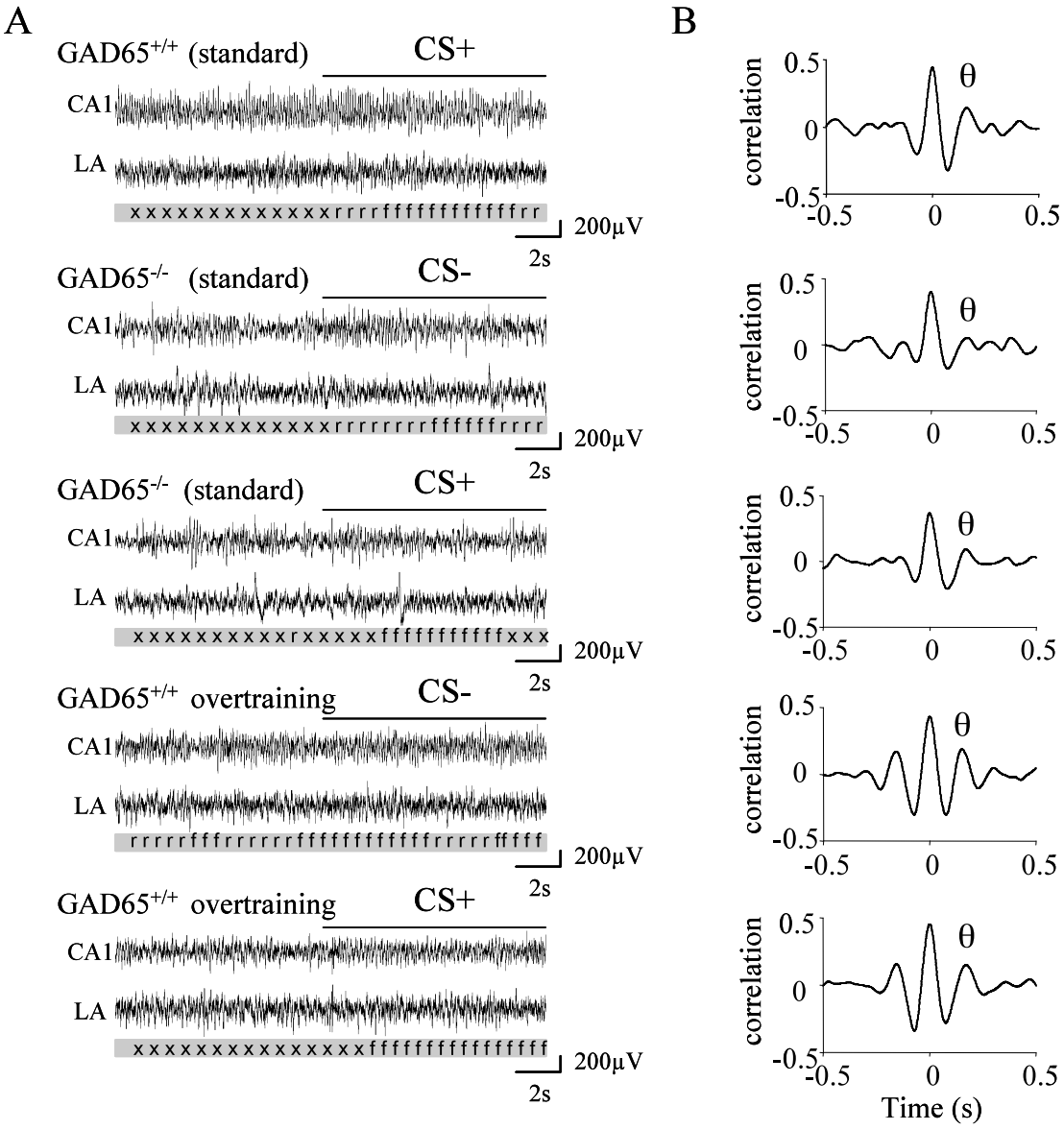


Figure 3.3.4. **Network activity during fear memory retrieval and generalization**. (A) Representative original traces from the hippocampus and amygdala of a standard conditioned GAD65$^{+/+}$, a standard conditioned GAD65$^{-/-}$, and an overtrained GAD65$^{+/+}$ animal during pre-stimulus and (CS+, CS-) stimulus phases with freezing behavior. x, exploration; r, risk-assessment; f, freezing. (B) Averaged cross-correllograms (θ, peak theta synchronization) note, the low theta peak in a GAD65$^{-/-}$ mice. ** p<0.01, * p<0.05, in the indicated comparisons. Values are mean+SEM.

I considered that this result may have been confounded by the reduced freezing behavior of GAD65$^{-/-}$ compared to GAD65$^{+/+}$ and overtrained animals. I therefore focused my attention further on the analysis of network activities during freezing episodes only, which are shown by GAD65$^{+/+}$ mice during the CS+ tone presentation and by GAD65$^{-/-}$ mice and overtrained GAD65$^{+/+}$ mice during both CS+ and CS- tone presentations. During these freezing periods, amygdalo-hippocampal theta synchronization was different between groups ($F_{4.37}$=3.715; P<0.01), as GAD65$^{-/-}$ animals showed generally lower cross-correlation levels (0.12±0.06 during the CS- and 0.15±0.04 during CS+) than the other groups (Fig. 3.3.5B). In particular, theta synchronization in GAD65$^{-/-}$ mice during generalized freezing to the CS- tone presentation was significantly lower than that of overtrained GAD65$^{+/+}$ mice freezing to the CS- (0.22±0.05; P<0.002) or CS+ (0.18±0.04; P<0.035). They were also lower than those of standard-conditioned GAD65$^{+/+}$ mice during CS+ freezing (0.19±0.05; P<0.009). The reduction of theta synchronization of GAD65$^{-/-}$ mice during the CS+ tone presentation, in contrast, failed to reach significance level.

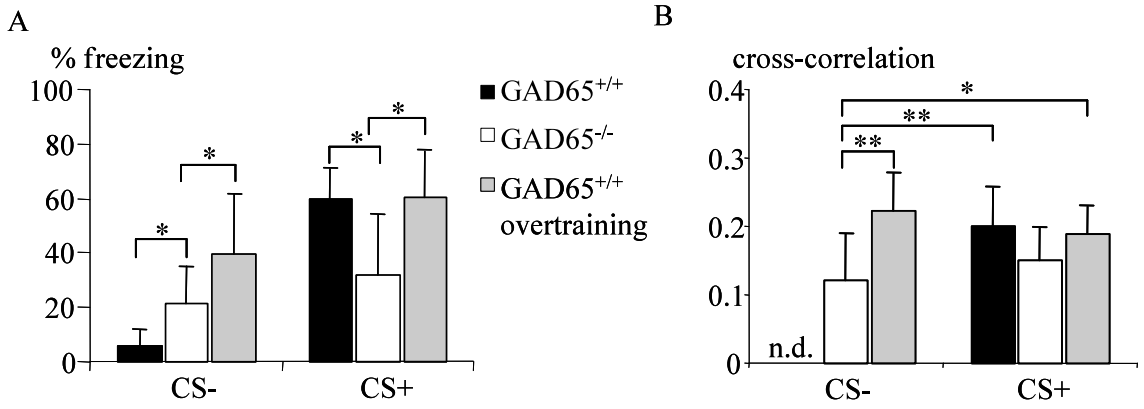


Fig.3.3.5. **Network activities during freezing episodes** (A) Behavioral analysis of the recorded animals. (B) Theta cross-correlation during episodes of freezing. ** p<0.01, * p<0.05, in the indicated comparisons. Values are mean+SEM.

3.3.3. Discussion.

In this study I present evidence for an involvement of the 65kD isoform of the GABA synthesizing enzyme glutamic acid, GAD65, in consolidation and generalization of conditioned fear. Firstly, I demonstrate that a deficiency in GAD65 results in a disturbance of stimulus-specificity during long-term fear memory consolidation and secondly, I describe network activity patterns that are associated with fear memory generalization in GAD65 mutant and wild type mice. GAD65 mRNA regulation in the amygdala at 24 h. after fear conditioning (Pape and Stork, 2003. Bergado-Acosta *et al*., 2008) strikingly coincides with a long-term memory phase that is dependent on the functional integrity of the amygdala (Suzuki *et al*., 2004) and involves the integration of amygdalar neurons into extended network activities (Narayanan *et al*., 2007). In light of the differential information processing through subsets of GABA neurons, reduced GABAergic function might thus be involved in the fine-tuning of long-term information storage, and may contribute to the pronounced and lasting reduction of extracellular GABA levels that can be observed in the amygdala during fear memory retrieval (Stork *et al*., 2002).

The data confirm previous observations of a reduced freezing response in $GAD65^{-/-}$ mice to an auditory fear cue, and extend it by showing a pronounced, context-independent intramodal generalization of their fear memory. Strikingly, this phenotype was only observed in (24 h. and 14 d) long-term, but not (30 min) short-term memory retrieval, indicating that it may result from a disturbance in the fear memory consolidation process. In fact, while wild type littermates increased their stimulus-specific response during consolidation by increasing freezing to the CS+ from STM to LTM, GAD65 null mutants not only failed to increase their response to the CS+ but instead increased freezing to the CS-. Moreover, while $GAD65^{+/+}$ mice showed a context-specific generalization to the CS-, the response of $GAD65^{-/-}$ mice was indistinguishable in the neutral and shock context. In fact, all genotypes showed an ambiguous fear-related behavior with low freezing and high risk-assessment scores in the

shock context, indicating a normal behavioral response of GAD65 mutants to situational reminders after cued conditioning. This is in agreement to previous observations in wild types (Laxmi *et al*., 2003) and with the normal performance of GAD65$^{-/-}$ mice in two hippocampus-dependent tasks, passive avoidance and water maze navigation (Asada *et al*., 1996).

Although GABAergic local circuit neurons comprise only 10-20 % of the neuronal population in the lateral and basolateral nuclei of the amygdala, they tightly control the spontaneous and evoked activity of projection neurons in this region (Rainnie *et al*., 1991) and govern the pattern of sensory evoked responses and the activity of the synaptic network (Lang and Pare 1997a;b; Szinyei *et al*., 2000). On the one hand, reduced inhibitory control in the amygdala is typically expected to enhance LTP and to facilitate Pavlovian fear conditioning, whereas enhanced inhibition has opposite effects (Muller *et al*., 1997; Wilensky *et al*., 1999). Pharmacological and genetic reduction of presynaptic inhibition at glutamatergic terminals unmasks non-associative, homosynaptic LTP in the basolateral amygdala and evokes generalization of auditory cued fear memory (Shaban *et al*., 2006). In the light of these findings it is also interesting that spike-timing dependent LTP in the amygdala is tightly controlled by the level of GABAergic inhibition (Shin *et al*., 2006). GAD65$^{-/-}$ mice display deficits in the posttetanic increase of inhibition following afferent stimulation in slice preparations of the amygdala or hippocampus, with spontaneous activity remaining unaffected (Tian *et al*., 1999).

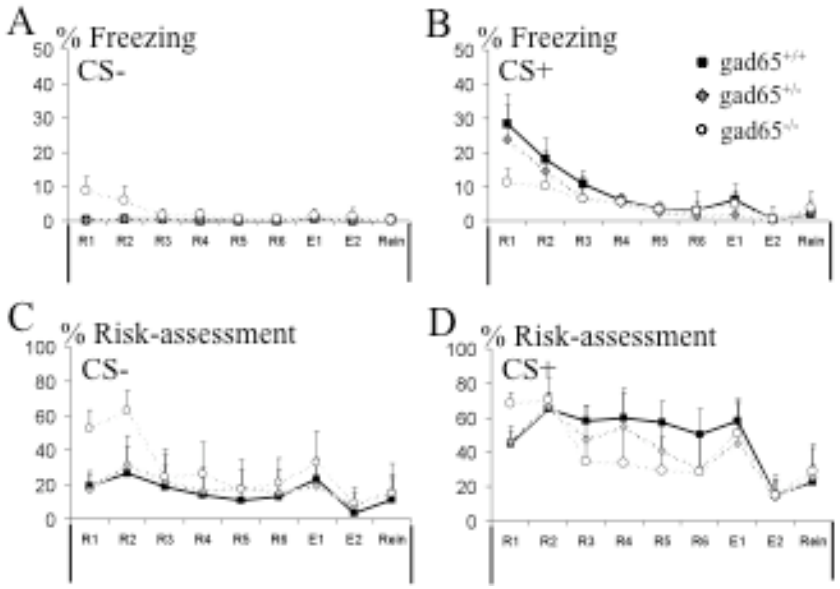
I therefore began to address the network activities that may be involved in these functions by analyzing activity patterns in the amygdala of GAD65$^{-/-}$ mice during fear memory retrieval and their incorporation into larger ensembles known to act within the amygdalo-hippocampal system (Seidenbecher *et al*., 2003). To investigate whether the observed network activities also translate to experience-dependent generalization of fear memory and to be able directly compare network activities of generalized fear memory during the CS- between GAD65$^{+/+}$ and GAD65$^{-/-}$ mice, we included a group of overtrained wild type mice to this analysis. In

agreement with previous observations (Seidenbecher *et al*., 2003, Narayanan *et al*., 2007) I observed a pronounced expression of 4-8 Hz type2 theta activity in the amygdala and hippocampus of standard-conditioned GAD65$^{+/+}$, standard-conditioned GAD65$^{-/-}$ or overtrained GAD65$^{+/+}$ mice. Moreover, we could observe considerable synchronization of theta activities between amygdala and hippocampus during both CS- and CS+ -induced freezing episodes. However, reduced levels of theta synchronization were observed in generalizing GAD65$^{-/-}$ mice compared to overtrained GAD65$^{+/+}$ mice during the CS-. It is important to point out that in wild type mice increased theta synchronization appears to be specific for a long-term memory stage around 24 h after training (Narayanan *et al*., 2007), which is coincident with the reduction of GAD65 gene expression in the amygdala. Thus theta synchronization clearly is not required for the retrieval or expression of conditioned fear *per se*, but likely represents a correlate of amygdalo-hippocampal information processing during this late stage of fear memory consolidation. My data now suggest that substantial differences exist in information processing in the amygdalo-hippocampal pathway during the (context-independent) expression of generalized fear memory in GAD65$^{-/-}$ mice and its (context-dependent) expression in overtrained GAD65$^{+/+}$ mice.

3.3.4. Fear extinction.

The GABAergic system moreover has been implicated in the extinction of fear memories (Berlau and McGaugh, 2006; Chhatwal *et al*., 2005). Fear extinction is a gradual process by which the conditioned response decreases after several CS presentations without US reinforcement (Myers and Davis, 2002; Maren and Quirk, 2004). Extinction learning takes place in three phases: acquisition, consolidation, and retrieval (Quirk and Mueller, 2008). Today there is considerable interest in those processes because they could have a direct impact on the development of new therapies against recurrent fear and anxiety disorders (Pine *et al*., 2009; Foa, 2006) and drugs that could facilitate the extinction learning hence are of

particular clinical relevance (Davis *et al*., 2006a; Davis *et al*., 2006b). Infusion of the $GABA_A$ agonist muscimol in the BLA, for example, reduces fear expression (Blair *et al*., 2005; Muller and Fendt, 2006), and facilitates extinction consolidation (Akirav *et al*., 2006). The role of GABA in the expression of extinction highlights the role of inhibitory networks in this process (Quirk and Gehlert, 2003). As a result, I decided to explore the extinction process in the GAD65 mice. For this experiment I used the GAD65 mutants, which underwent cued fear conditioning 24 before. Extinction training consisted of 5 more retrieval session (R2 through R6) identical to the first, performed at 30min intervals. 24 h later, long-term extinction memory was tested in two test sessions (E1 and E2). Then fear memory was reinstated through an unspecific stressor (presentation of a rat for 5min; Rein).



Fear memory extinction of GAD65 mice. (A) Generalized fear response of the $GAD65^{-/-}$ can be normalized by extinction training. (B) Cued fear responses decreased upon training and keep low after 24 h. (C,D) Risk-assessment respond during extinction decreased after R2 for the CS-, At the same time, CS+ responding of $GAD65^{-/-}$ mice was also normalized by the extinction training, reaching similarly low levels of freezing and risk assessment on E1 and E3, as well as following reinstatement.

The results obtained remarke the effectiveness of extinction training to treat recurrent fear. GAD65$^{-/-}$ mice could successfully decrease the generalized freezing and risk-assessment response to the CS- after two retrieval sessions that remain low on the next day. The cued freezing respond was also extinguished; but the GAD65$^{-/-}$ mice have small respond from R1 making difficult to conclude if they actually remember. The risk-assessment behavior during cue retrieval shows a very interesting picture, GAD65$^{+/+}$ do not decrease their response over the whole training session. Further they have high level of risk assesment next day and this is only abolishing after E1 retrieval. On the other hand the GAD65$^{-/-}$ that started with high levels of arousal are able to reduce it after the second retrieval R2, but is elevated in the E1. Behavior of the GAD65$^{+/-}$ mice is comparable to GAD65$^{+/+}$ except for extinction training, where risk-assesment responses where at an intermediate level between GAD65$^{+/+}$ and GAD65$^{+/-}$.

Like in fear conditioning, the amygdala seems to play a critical role in fear extinction (Phelps *et al*., 2004; Davis *et al*., 2003; Figueroa and Quirk, 2005). Especially a population of GABA intercalated cells, positioned between BLA and CeA have been postulated to provide a substrate for expressing and storing extinction (Pare *et al*., 2004; Quirk *et al*., 2003). This interneurons receive inputs from the medial part of the prefrontal cortex (mPFC) (Mcdonald *et al*., 1996; Ghashghaei and Barbas, 2002) a region that in humans is important for estimating the salience of emotional stimuli and for selecting correct actions (Ochsner and Gross, 2005; Sotres-Bayon *et al*., 2006). The human mPFC shows robust activity measured by fMRI during fear extinction (Phelps *et al*., 2004; Delgado *et al*., 2008). In rodents, the mPFC projects to the amygdala (Mcdonald *et al*., 1996; Vertes, 2004), and mPFC neurons show fear extinction-related increases in activity (Milad and Quirk, 2002; Burgos-Robles *et al*., 2007), as well as gene expression changes (Herry and Mons, 2004; Santini *et al*., 2004; Quirk *et al*., 2006). However lesion studies are contradictory, Quirk *et al*., (2000) showed that rats with mPFC lesions can learn to extinguish fear, but have difficulties recalling the extinction memory 24

hours after training. In contrast (Garcia *et al*., 2006) exposed that electrolytic lesions of the mPFC do not interfere with extinction of conditioned fear. More recently, it was found that inactivation of PFC reduced learned fear to the cue and to the context but had no effect on innate fear, suggesting that PFC integrates auditory and contextual inputs and regulates expression of fear memories (Corcoran and Quirk, 2007).

Remarkable is the finding that lesions of the mPFC 200 days following trace fear conditioning disrupt remote memories while dorsal hippocampus (DH) lesions after 1 day disrupt recently encoded trace fear memories (Quinn *et al*., 2008). In fact, extinction is a context-dependent process and therefore it is not surprising that it needs the hippocampus (Bouton *et al*., 2006; Ji and Maren, 2007). Electrolytic lesions of the DH disturb the context specificity of extinction, independent of whether lesions were made before training or after extinction (Ji and Maren, 2005). The hippocampus modulates the responses of LA neurons to conditioned tones during fear extinction, supporting the idea that LA is a locus of hippocampal modulation and suggesting that this modulation is through inhibitory network within the LA (Hobin *et al*., 2003; Maren and Hobin, 2007). In essence, this shows that local GABAergic interneurons are important targets for the neuromodulatory systems that impinge on fear memory formation.

3.4. NPS effects on fear conditioning.

Paper title: Identification of a neuropeptide S responsive circuitry shaping amygdala activity via the endopiriform nucleus.

Authors: Meis S., Bergado-Acosta J.R., Yanagawa Y., Obata K., Stork O., and Munsch T.

Published in: PLoS ONE. 2008 3: e2695.

Contribution: Design, conduction, statistical analysis and interpretation of the behavioral experiments.

3.4.1. Background and Rationale.

A number of neuropeptide systems are involved in the control of fear and anxiety responses and its effects in the brain prepare the organism for threat, and modulate attention, vigilance and memory (Wiedenmayer, 2004). For example the neuropeptide S (NPS) has recently been identified as a factor that control food intake (Beck *et al*., 2005), increase locomotion and arousal, and attenuates anxiety-like behavior (Xu *et al*., 2004). The human NPS is a 20-residue peptide (SFRNGVGTGMKKTSFQRAKS) the N-terminal serine residue; give the name to this novel neuropeptide (Roth *et al*., 2006). NPS is expressed in three brainstem nuclei (locus coeruleus, lateral parabrachial nucleus and principle trigeminal nucleus) as well as in neurons of the medial amygdala and dorsomedial hypothalamus (Xu *et al*., 2007). NPS has been identified as the endogenous agonist for the orphan G-protein coupled GPR154 receptor (NPSR) (Xu *et al*., 2004). This receptor is believed to mediate mobilization of intracellular Ca^{++} and increase of cAMP, suggesting that NPS may enhance neuronal excitability (Reinscheid *et al*., 2005). NPSR mRNA is expressed in various stress-related brain regions, such as the amygdala, hypothalamus, raphe nuclei, and ventral tegmental area (Xu *et al*., 2004).

Strikingly, NPS receptors are also highly expressed in the endopiriform nucleus (EPN) (Xu *et al*., 2007) which is known to connect to the enthorinal and perirhinal cortices as wells as the amygdalo-hippocampal transition area and the basolateral amygdala (Majak *et al*., 2004; Behan and Haberly, 1999) Moreover stimulation of the ventral EPN can evoke excitatory postsynaptic potentials in the BLA (Gean and Chang, 1992) and is able to drive oscillatory activities in the amygdala (Ponomarenko *et al*., 2003). Our colleagues Dr. Susanne Meis and Dr. Thomas Munsch observed that direct infusion of NPS in EPN activates an inward current in 20 % of neurons and evokes an increase of glutamatergic excitation in this nucleus. They also showed that, excitation of the EPN modulated BLA activity, characterized by a general increase of GABAergic inhibition and enhancement of spike activity in a subset of BLA projection neurons. The disconnection of BLA and EPN prevented the NPS-induced increase of sIPSCs in BLA projection neurons. Therefore, we hypothesized that NPS application into the EPN may be able to interfere with specific aspects of fear memory retrieval that are carried by synchronized network activities in amygdalo-hippocampal pathways. To investigate this possibility I investigated the effect of local NPS application to the mice EPN on the retrieval and expression of conditioned fear.

3.4.2. Results.

Injection of NPS to the EPN resulted in a reduction of contextually conditioned fear behavior without apparent deficits in auditory cued fear memory or in general anxiety level (Fig. 3.4.1). In detail, during the retrieval of contextual fear memory, NPS treatment had a significant effect on the expression of typical rodent defensive behaviors, i.e. freezing ($F_{2.37}$=4.679; P=0.016) and risk assessment ($F_{2.37}$=11.084; P=0.000). Planed comparison revealed a reduction of freezing (1.45±2.51 % compared to 15.09±16.32 % for vehicle treatment, P=0.009; Fig. 3.4.1), as well as risk assessment behavior (15.35±11.81 % compared to

37.48±15.91 % in the vehicle group, P=0.001; Fig 3.4.1) in the 0.1 nmole NPS treatment group. Animals treated with the lower dose of 0.01 nmole NPS failed to show significant change from vehicle treated controls (freezing: 13.28±14.86 %; risk assessment: 41.75±18.57 %). NPS treatment at either dose failed to significantly affect freezing ($F_{2.37}$=1.60; P=0.216) or risk assessment behaviour ($F_{2.37}$=0.425; P=0.657) during auditory cued fear memory retrieval, although a trend for somewhat lower freezing values was observed in both NPS treatment groups (24.52±12.51 % at 0.1 nmole and 31.91±19.31 % at 0.01 nmole, compared to 36.46±19.66 % for vehicle controls; Fig 3.4.1). Paired comparisons values CS- Freezing: 0.1 nM 7.05±10.57; 0.01 nM 12.30±10.94; vehicle 18.01±16.17 ($F_{2.37}$=1.659; P=0.205); Risk-assessment: 0.1 nM 36.79±14.66; 0.01 nM 48.28±16.95; vehicle 37.46±15.78 ($F_{2.37}$=2.071; P=0.141). No significant effect of NPS treatment was observed on anxiety-like behaviour in the elevated plus maze, as time on open arms ($F_{2.37}$=0.176; P=0.839), the % entries to open arms ($F_{2.37}$=0.297; P=0.744), and total number of arm entries ($F_{2.37}$=0.471; P=0.627) remained unchanged (Fig 3.4.1).

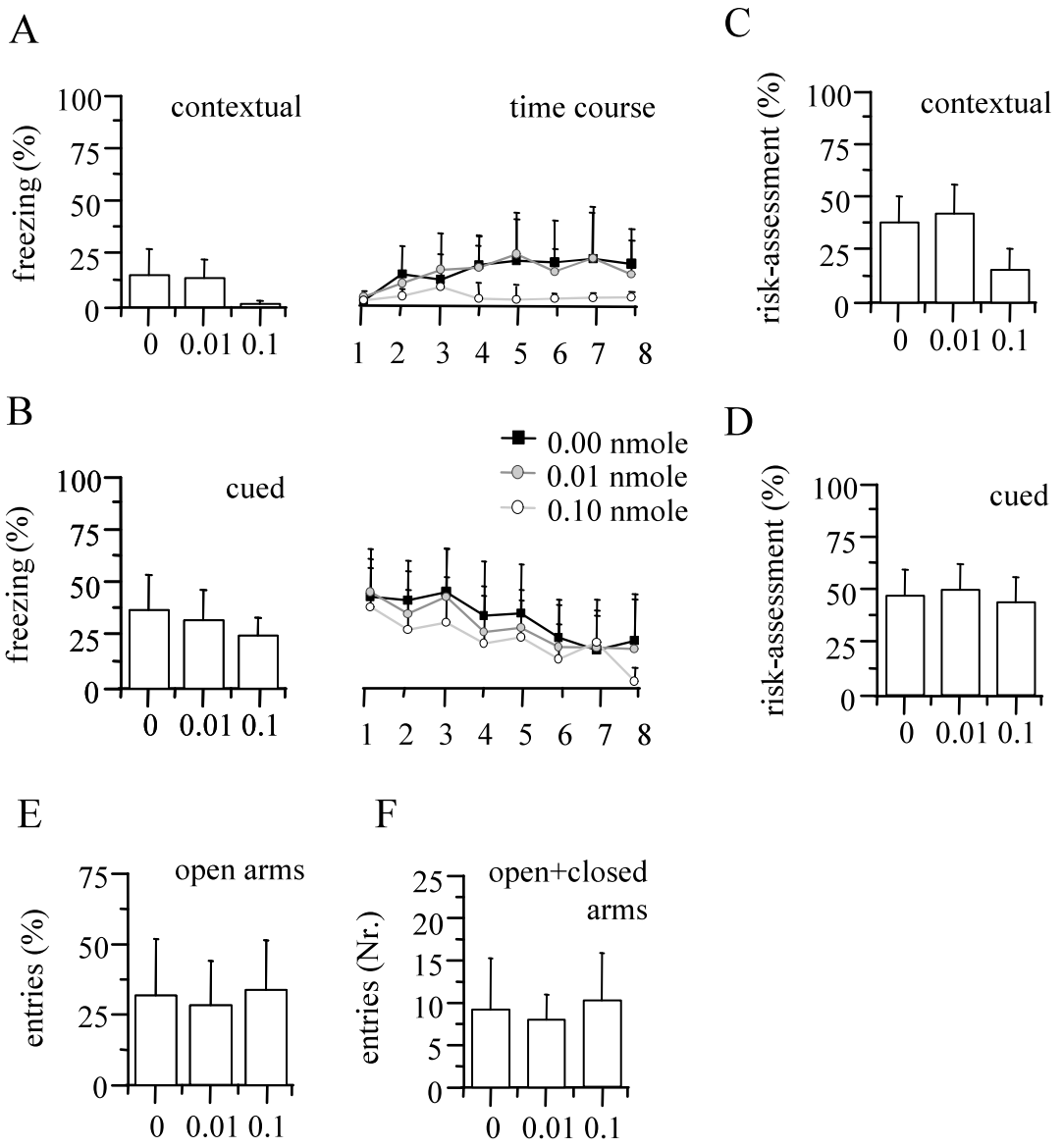


Figure 3.4.1. **Behavioral NPS effects.** (A) Reduction of contextual fear responding was observed upon NPS administration. 0.1 nmole to the EPN (n=14), compared to vehicle injected controls (n=12) and 0.01 nmole,(n=12). Time course analysis of conditioned freezing behavior revealed a continuous reduction during the contextual retrieval session. 1-8, test intervals analogous to the cued retrieval. (B) Cued fear memory retrieval, 1,3,5,7, conditioned stimuli (10s), 2,4,6,8 ISI (20s). (C, D) Reduction during contextual vs. cued memory retrieval was observed for, risk-assessment. (E, F) No change of anxiety-related behavior was apparent in an elevated plus maze. All values are mean+SEM; *, $p<0.05$; compared to vehicle, **, $p<0.01$.

3.4.3. Discussion.

NPS administration to the EPN in my experiments resulted in a selective disruption of contextual fear memory retrieval with reduction of both, freezing and risk-assessment. At the same time, auditory cued fear memory remained unaffected by the treatment, excluding the possibility of a general interference with the retrieval or expression of conditioned fear. By analyzing risk-assessment behavior and generalization of fear memory to a neutral acoustic stimulus (CS-) as within-task measures of anxiety-like behavior, I furthermore could exclude a potentially confounding general effect of NPS on anxiety level during the retrieval session. By testing plus maze behavior immediately prior to the fear memory, I could support this point and conclusively rule out a general effect of my treatment on anxiety-like behaviors as is has been previously seen upon intracerebroventricular injection at the dosage used (Xu *et al*., 2004). In turn, it may be concluded that the circuitry delineated in our study may not to be critical for such modulation of general anxiety through NPS.

Thus, my behavioral observations demonstrate that NPS is indeed able to selectively modulate fear memory functions mediated by amygdalo-hippocampal interactions, as predicted on the basis of the physiological and available anatomical neuro circuitry analysis. The behavioral change in my experiments is likely due to a local effect of NPS through the activation of NPS receptors in the EPN, although, I cannot rule out a spreading to the NPSR-endowed the lateral enthorhinal cortex. Further studies will be required to precisely delineate functionally distinct NPS circuits in the brain and their activation under different fear and anxiety conditions. For example it has been shown that intracerebroventricular infusion of NPS reinstates extinguished cocaine-seeking behavior in mice, suggesting that NPS receptors may be an important target for amygdala-mediated behaviors and drug abuse research (Pañeda *et al*., 2009). Recently Jüngling *et al*., (2008) showed that pre-training injection of NPS in the LA / BLA did not interfere with cue fear memory retrieval or extinction. However pre-retrieval injection did have an effect in fear extinction. As I described above, extinction is a

hippocampus dependent process, therefore this results are some how correlate to our deficit in contextual fear memory by pre-retrieval injection. These authors also remarked the importance of GABA interneuron excitation in the amygdala as possible target of NPS modulation.

4. Synthesis & general discussion.

4.1. Role of GABA in fear memory-related network activities.

Over the last two decades, the circuitry, neurotransmitter systems and some cellular and molecular processes that are involved in fear memory formation have been identified (Adamec, 2000; Buzsáki, 2002; Paré *et al*., 2002; Pelletier and Paré, 2004; Santini *et al*., 2004; Stork *et al*., 2004). Evidence further suggests that fear conditioning also induces synchronization of neuronal activity of the LA neurons (Pare and Collins 2000) and coherent theta-rhythm activity in the amygdalo-hippocampal pathways (Seidenbecher *et al*., 2003). However, the molecular and cellular processes that underlie these network activities are not yet understood. In my studies I began to investigate these functions, with a focus on the role of GABAergic interneurons.

As first step I investigated the involvement of such theta synchronization in distinct phases of fear memory consolidation and the modulation of this process by stress (Narayanan *et al*., 2007; Albrecht *et al*., in revision). My data demonstrate an involvement of LA / CA1 theta synchronization in the retrieval of fear memory at long-term stages of cued and contextual, but not at short-term stages. They also reveal an association of reduced theta synchronization in NCAM deficient mice with their inability to enhance context fear memory upon stressful training. In general we can infer from these data that theta synchronization is not required for the expression of conditioned freezing *per se*, as this behavior occurs in wild type mice during short term memory stages and in $NCAM^{-/-}$ with standard training in spite of reduced synchronization levels. Hence it is likely that this network activity provides a means of information processing during the expression of conditioned fear.

Memory traces are maintained within steady networks, which integrate and update past traces with new ones (de Vries and van Slochteren, 2008). Theta may be a marked signal of one effective recall situation where explicit items match with previous ones, but is not exclusive

for context or cued signals. The observation that similar levels of this synchronization are seen in both contextual and cued fear memory retrieval (Seidenbecher *et al*., 2003) lends itself to the conclusion that here context information (foreground context retrieval or the background context associated with the cue) may be processed and compared to the retrieval context. In addition, the increase of theta towards the end of the acoustic stimulus suggests that temporal information is also involved.

Both context and temporal coding are key functions of the hippocampus. My observation in $NCAM^{-/-}$ mice adds to this the important aspect that the information flow is bidirectional in this pathway and that information about stimulus salience and stress level, encoded by the amygdala, appear to be relayed into the hippocampus during theta phase synchronization. Null mutation of the NCAM gene results in deficits in cued and contextual fear conditioning (Stork *et al*., 2000a). These mice are also characterized by increased anxiety-like behavior associated with a hypersensitivity of the serotonergic system (Stork *et al*., 1999) and show impaired sensitization of the startle response (Plappert *et al*., 2006). Reduction of NCAM expression in the adult hippocampus of conditional deficient mice reduces precision of spatial learning as well as LTP and LTD in the CA1 subfield of hippocampus, thus remarking the role of NCAM in the regulation of synaptic plasticity despite developmental abnormalities (Bukalo *et al*., 2004).

Building on the so specified function of theta synchronization, I began to address the role of GAD65-mediated GABA synthesis in fear memory and associated theta phase synchronization. GABAergic mechanisms are critical for conditioned and unconditioned fear and to a large extend these functions can be attributed to the BLA (Pesold and Treit, 1995; Sanders and Shekhar, 1995). As the BLA serves as entry site for hippocampal information to the amygdala during contextual fear conditioning (LeDoux, 2000), it was highly warranted to investigate potential effects of GABA deficits on theta synchronization. My data suggest that fear memory in $GAD65^{-/-}$ mice occurs independent of the retrieval background and is

associated with reduced theta synchronization. We know from previous studies that GABA interneurons exert a fine-tuning control over projection cells and their plasticity, and are themselves able of long-term potentiation as well as depression in synaptic transmission. Particularly in the amygdala, the GAD65 gene is regulated after fear conditioning (Pape and Stork, 2003), and the absence of the enzyme provokes a general decrease of freezing and increase in risk-assessment like behavior. As demonstrated by mutant mice deficient in GAD65, synthesis reduction of GABA causes context-independent generalization and reduction of long-term, but not short-term, cue-specific fear memory. Thus, GAD65-mediated GABA synthesis may well be involved in both, associative and non-associative (Siegmund and Wotjak, 2007) aspects of plasticity in the amygdala and hippocampus during fear memory formation.

It should be considered that different levels of GAD65 expression during consolidation, or reduction in specific cell types may be important for stimulus specificity. A complete loss of GAD65 activity in the mutant mice may thus exaggerate the effects of endogenous regulation in a gene-dosage dependent manner. The lack of generalization in $GAD65^{+/-}$ mice appears to argue against this, but it should be considered that adult heterozygots display normal GABA content in the amygdala and hippocampus (Stork *et al*., 2000). Secondly, GAD65 regulation and function may differ between subpopulations of GABA interneurons, which are characterized by particular morphology, physiology and the expression of neurochemical factors (Freund and Buszaki, 1996; Frenois *et al*., 2005; Mascagni and McDonald, 2007; Muller *et al*., 2007). GABA interneurons are thought to mediate specific aspects of fear memory-related information processing within the amygdala (e.g., Quirk *et al*., 2003, Azad *et al*., 2004, Marowsky *et al*., 2005) and hippocampus (Crestani *et al*., 1999, Gafford *et al*., 2005). Thirdly, if compensatory mechanisms are active in the GAD65 null mutants, the inability of these animals to change GABAergic function via regulation of GAD65 expression may be important. Finally, it must be considered that GAD65 mutation may affect network

properties in the amygdala and hippocampus even before training, thereby interfering with cellular processes that are triggered during the memory acquisition phase or non-specifically increasing the responsiveness to aversive stimuli by enhancing general anxiety levels (Stork *et al*., 2000). However, the latter explanation appears unlikely since immediate post-training freezing, which is a function of training intensity and aversiveness (Laxmi *et al*., 2003) was not altered in the GAD65 mutant mice.

4.2. Mechanisms that control GABAergic transmission via GAD gene regulation.

Fear conditioning activates CREB which in turn drives the expression of many genes, such as c-Fos, which has repetitively been observed in the amygdala following fear conditioning (Campeau *et al*., 1991; 1997; Milanovic *et al*., 1998; Rosen *et al*., 1998). CREB gene products further include the transcription factors (nerve growth factor-inducible A) NGFI-A / Zif268 and NGFI-B (Rosen *et al*., 1998). The induction of both transcription factors in the amygdala can be prevented through anxiolytic pre-treatment or through blockage of conditioning with APV (Malkani and Rosen, 2000; 2001). CREB phosporylation is also linked to histone acetylation through CREB binding protein (CBP), which itself is required for fear conditioning (Oike *et al*., 1999). CBP works as a platform for recruiting other transcriptional components and as a histone acetyltransferase (HAT) (Korzus *et al*., 2004). Several mechanisms have been identified that might be involved in the short-term and long-term regulation of GAD and thereby modulate fear behavior. Firstly, genetic variation has been recently observed that determines expression levels of GAD65 and thereby may generally determine the strength of inhibitory transmission in the brain. However, so far only pancreatic consequences of this polymorphis have been investigated. Hence it may be possible to

translate some of the findings made in GAD65 mutant mice into humans. (Boutin *et al*., 2003).

Second, epigenetic factors regulate GAD67 expression directly, and potentially via Dlx5 also GAD65 (Huang *et al*., 2007). Epigenetics are post-translational modifications of deoxyribonucleic acid (DNA) and histones. This results in durable alterations in chromatin structure, which produces lasting alterations in gene expression and consequently behaviors (Levenson and Sweatt, 2005). Recent studies demonstrated that DNA methylation is dynamically regulated following contextual fear conditioning (Miller and Sweatt, 2007; Levenson *et al*., 2006; Miller *et al*., 2008). DNA methyltransferases (DNMT) is upregulated in the rat hippocampus 30 min. after training (Miller and Sweatt, 2007). Moreover intra CA1 DNMT inhibitors induced deficits in memory consolidation along with deficits in LTP that can be rescued by pharmacological increase of histone acetylation prior to DNMT inhibition (Miller and Sweatt, 2007; Miller *et al*., 2008). Contextual fear conditioning also leads to a rapid time-dependent increase in histone H3 phosphorylation in area CA1 (Chwang *et al*., 2006). In the amygdala, direct infusion of the histone deacetylase (HDAC) inhibitor trichostatin A into the LA or BLA significantly enhanced the formation of fear-potentiated startle memory (Yeh *et al*., 2004). A different study shows that valproic acid enhances long-term memory for both acquisition and extinction of cued fear, remarking the importance of HDAC inhibitors as adjuncts of behavior (Bredy and Barad, 2008). Taking together these observations suggest that DNA methylation may work in concert with histone modifications to regulate the expression of genes that support memory formation and behavior plasticity.

Third, the regulation of fear and anxiety through GABA interneurons importantly include estrogen hormones, which are strong modulators of behavior, affecting functions like reproduction, emotion, and cognition (Walf and Frye, 2006). Two types of estrogen receptors named, α and β have been identified; they are members of the steroid nuclear receptor family that regulate gene transcription (Keightley, 1998). Steroids receptors can be localized in the

plasma membrane as well, coupling with G proteins directly or indirectly (Hammes and Levin, 2007). Steroid response include transcriptional and non-transcriptional actions; directing both the rapid and delayed actions of estrogen (Levin, 2005; Levin, 2008). High expression of α and β estrogen receptor isoforms are observed in limbic areas, specially in the extent medial nucleus of the amygdala and BNST. In the hippocampus immunohistochemistry studies suggested that only the α receptor is sparsely expressed in specific subfields (Pérez *et al*., 2003; Mitra *et al*., 2003). However double labeling fluorescent immunohistochemistry exposed that the estrogen β receptor colocalizes with GABAergic-associated calcium-binding protein, parvalbumin (PV) in the amygdala, basal forebrain, and hippocampal formation (Blurton-Jones and Tuszynski, 2002). Similarly double-label immunohistochemistry for estrogen receptor α and GAD revealed that many cell expressing this receptor, especially in the dorsal hippocampus, are GABAergic. (Hart *et al*., 2001).

Estradiol seems to regulate GABA tone in a temporal manner, as a decrease in the amount of GAD65-immunoreactive interneurons is observed in the CA1 region of the hippocampus 24 hours after estrogen treatment that is accompanied by IPSC decay but it recovers by 48 hours after a second estrogen treatment (Rudick and Woolley, 2001). Nakamura and coworkers (2004) explored this idea and found the basal number of GAD65-immunoreactive signals in fusiform cell bodies, varicosities and axon terminals decreased onto CA1 pyramidal cells 10 days after ovariectomy as compared with 3 days. In long-term ovariectomized rats GAD65 mRNA levels increased within 24 hours after estradiol treatment, followed by a subsequent increase in GAD67 mRNA levels. (Nakamura *et al*., 2004). These observation might explain previous findings by Murphy and Segal (2000), who blocked the induction of dentritic spines in CA1 region of the hippocampus (Woolley and McEwen, 1992; Murphy and Segal, 1996, Murphy *et al*., 1998) by application of progesterone that is known potentiate the inhibitory actions of $GABA_A$ receptors (Majewska *et al*., 1986; Rupprecht *et al*., 1996).

Further, fear related behaviors and associated plasticity seem to have strong sexual dimorphisms (Maren *et al*., 1994; Edinger *et al*., 2004). Especially ovarian steroids may be responsible for such differences (Anagnostaras *et al*., 1998). Ovariectomized female rats, for instances, show freezing levels equivalent to sham-group or male rats. The replacement of estrogen level in this animals reduced contextual freezing behavior and hippocampal LTP (Gupta *et al*., 2001). Moreover, increased anxiety in the open field and elevated plus maze is observed in female estrogen receptor β mutant mice. These effects were associated with a reduction in BLA synaptic plasticity induction for the mutant females (Krezel *et al*., 2001). Consistently, β estrogen receptor knockout mice present memory deficits in contextual fear conditioning and impaired hippocampal CA1-LTP induced by theta burst (Day *et al*., 2005). In summary the action of this hormones suggest that they downregulated GABA related factors at first, allowing thus plasticity in projection neurons. But once the new synaptic contacts are formed they increased GABA tone to normal level, stabilizing the balance between excitation and inhibition.

Fourth, it is critical to investigate how GABAergic network mechanisms in the amygdala may be controlled by environmental stimuli. Local GABAergic interneurons that contain diverse neuromodulatory substances are best sites for the study of sensory and affective information in the amygdala and their modulation through processes of memory formation. Various neurotransmitters, neuropeptides and hormones have been implicated in the acute response to stress and in the formation of fear memories (Charney, 2004). For instance, many kinds of stressful stimuli produce increases in brain noradrenergic function (Gallagher *et al*., 1977). Fear memory formation is dependent on an acute increase of norepinephrine release in the BLA (Ferry *et al.,* 1999; LaLumiere *et al*., 2003) and hippocampus (Ji *et al*., 2003) activating local α1- and β-adrenoreceptors (Schramm *et al*., 2001; Davies *et al*., 2003). This response results from an activation of the nucleus of the solitary tract (Williams and McGaugh, 1993), which in turn triggers norepinephrine release in the amygdala, e.g., following foot-shock

exposure (Galvez *et al*., 1996; Williams *et al*., 2000; McGaugh, 2002). In addition to this, it has been recently suggested that adrenergic modulation of local interneurons may contribute to the formation of fear memory by gating LTP at thalamo-amygdala synapses (Tully *et al*., 2007). This effect maybe mediated by presynaptic α1A adrenoceptors that facilite GABAergic inhibition by decreasing the excitability of local interneurons (Braga *et al*., 2004). Norepinephrine also stimulates the release of somatostatin in amygdala interneuron populations (Epelbaum *et al*., 1981; McDonald and Mascagni, 2002).

In fact, neuromodulatory innervation to the LA / BLA is highy selective for subpopulations of GABA interneurons. Serotonergic (5-HT) afferences, for example, act on parvalbumin-negative, cholecystokinin (CCK)-positive interneurons (Bloom and Morales, 1998; Marsicano and Lutz, 1999). The release of serotonin in the amygdala under stressful conditions may discriminate between escapable and inescapable stress, hence of particular relevance for the performance of classical fear conditioning (Amat *et al*., 1998). Lesion and autoinhibition of serotonergic neurons in the median raphe nucleus reduce contextual fear conditioning (Avanzi and Brandao, 2001). Consequently, blockade of 5-HT_{1A} receptors as the major postsynaptic 5-HT receptor type prevents cued fear conditioning (Stiedl *et al*., 2000) and disturbs LTP in the LA / BLA (Pollandt *et al*., 2003). Interestingly, serotonergic transmission in close cooperation with local GABAergic interneurons mediates stress effects on afferent sensory transmission to the LA (Stutzmann *et al*., 1998; Stutzmann and LeDoux, 1999).

Under stressful conditions there is also an important release of dopamine (DA) in the amygdala (Inglis and Moghaddam, 1999). Its effects may be related to alterations of neural excitability in the LA and afferent input from the medial prefrontal cortex (mPFC) (Grace and Rosenkranz, 2002; Seamans and Yang, 2004; Bissiere *et al*., 2003). Uncontrollable stress activates mPFC DA release (Ventura *et al*., 2002) and inhibits nucleus accumbens DA release (Cabib *et al*., 2002). Lesion of amygdala before and after fear conditioning blocks stress-induced mPFC DA metabolic activation, suggesting an amygdala-mediated control of stress-

induced DA activation and integrated behavioral and neuroendocrine components of the stress response (Goldstein *et al*., 1996). Blockade of amygdala dopamine release by inhibition of dopamine receptors type-2 (D2) in the ventral tegmental area decreases fear memory retrieval (Greba *et al*., 2000; Nader and LeDoux, 1999), and antagonism of both D1 and D2 receptor in the amygdala itself blocks the acquisition and expression of conditioned fear (Lamont and Kokkinidis, 1998; Guarraci *et al.* 1999, Nader and LeDoux, 1999; Guarraci *et al*., 2000). DA application in amygdala coronal slices robustly increased the frequency of spontaneous inhibitory potentials (sIPSCs) in LA projection neurons and induced low-frequency (2–6 Hz) oscillatory activity of inhibitory circuits, suggesting that DA organize the activity of populations of interneurons in the LA (Lorétan *et al*., 2004). Dopaminergic afferences project mainly onto parvalbumin-positive interneurons in the BLA (Brinley-Reed and McDonald, 1999; Grace and Rosenkranz, 2002).

Fifth, the recent observation of NPS as a peptide that modulated the activity of the EPN provided a new clue. NPS produces anxiolytic-like effects by acutely reducing fear responses as well as modulating long-term aspects of fear memory, such as attenuation of contextual fear or enhancement of fear extinction (Pape *et al*., 2009). High frequency field oscillations have been observed in the EPN and BLA in freely behaving rats and it was suggested that this short-scale synchronous firing of subpopulations of projection neurons may organize the precise timing of multi-modal information flow in the BLA. NPS administration to the EPN in my experiments resulted in a selective disruption of contextual fear memory retrieval but not auditory cued fear memory. In vitro studies moreover indicated that, in BLA projection neurons, NPS stimulates GABA-mediated synaptic activity dependent on action potential propagation. It is likely that NPS, by orchestrating GABAergic network activities in the BLA, may control amygdalo-hippocampal interactions and the processing of contextual information during fear memory consolidation. It can be expected that future investigation of similarly specialized neuropeptidergic systems that target the amygdala, such as the orexin or the

oxytocin system (Sakurai, 2007), will help to further dissect the circuitries and GABA neuron populations that control specific aspects of fear memory formation.

These findings provide insight into mechanisms that underlie the development of fear and anxiety and identify genes that may represent genetic disposition factors. The understanding of these processes on a molecular level will be particularly important for our knowledge about the stability of fear and anxiety, and may guide future therapeutic approaches to anxiety disorders. In fact, classical fear conditioning has proven a useful model for understanding the psychological and pathological basis of emotional disorders, such as PTSD (Rau *et al*., 2005, Maren, 2005). Fear conditioning in patients with PTSD causes vivid recollection of traumatic events, autonomic hyperarousal and steel flashback associated with prior trauma (Charney 2004). In the ground of preclinical research animal models are used to look for compounds with therapeutic potential (Blanchard *et al*., 2003) and to study the neurobiological mechanics underlying anxiety behaviours (Adamec 2000). “The advantages of the mouse for behavioral studies include an extensive array of genetic technologies and an elaborate behavioral repertoire that can be used to create models of human disease”(Bućan and Abel, 2002).

5. Concluding Remarks and future aspects.

While providing conclusive evidence for the critical role of GAD65-mediated GABA synthesis in fear memory and associated network mechanisms, my studies have also raised new questions that need to be addressed in the future. As one important target, it should be clarified, which processes determine the observed temporal specificity of the theta synchronization? What are the cellular correlates associated with the increase of theta, and can different time windows be generated with appropriate training? For example, Narayanan *et al*., (2007b) could demonstrate that theta synchronization becomes reactived upon fear memory retrieval of remote memories, indicating an involvement of this network activity in memory reconsolidation.

Moreover, other behavioral states may utilize theta synchronization and it should be clarified how these relate to the fear memory induced network activity. For example, theta is also observed in amygdala and hippocampus during REM sleep. The relation of sleep and consolidation of fear memories have been shown that not sleep during the consolidation phase block memories. In a recent paper (Hegde *et al*., 2008) report a change in LA / CA1 synchronization during (REM) sleep in acutely stressed rats. It would be interesting to see whether a replay of fear conditioning information during REM sleep may be demonstrated by comparing signals (theta sleep vs. theta retrieval). Other frequency bands, such as the GABA-dependent gamma band should be analyzed for their potential involvement in fear memory formation. This approach is valid to different mutants mice, in that the effects of the mutation in the generation of rhythms can be studied in relation to the expression of behavior.

May be relevant to test out for activation of amygdala and hippocampus during situation with different stress levels. It may be possible to relate effect of GAD mutation to acute functions in particular brain region by rescue experiment with local injections of, e.g. tiagabine to increase the GABA content. Also, with the development of more sophisticated molecular

tools (Luo *et al*., 2002), it should become possible to produce a temporally and spatially restricted GAD ablation to this end. Hence it will be interesting to investigate the potential involvement of GAD65 in the stability of fear memories and their susceptibility to extinction (Sanga *et al*., in revision). GABA neurons are considered input stations for excitatory projections from the prefrontal cortex involved in the extinction of conditioned fear reactions (Rosenkranz and Grace, 2002; Quirk *et al*. 2003), and NPS effects have been related to the extinction of fear memories.

Finally, it must be considered that GABA interneurons represent a highly diverse population of cells, that is characterized by different morphological, physiological and neurochemical properties. Several neuropeptide co-transmitters, such as neuropeptide Y (NPY) have behaviorally relevant effects on the hippocampus and amygdala (Flood *et al*., 1989, Thorsell *et al*., 1999; Thorsell *et al*., 2000). Another neuropeptide typical of LA / BLA interneurons, is the somatostatin (SST). In a recent study an impairment of foreground and background contextual but not tone fear conditioning was shown in mice with targeted ablation of the somatostatin gene or upon acute systemic SST depletion with cysteamin. These deficits were associated with a decrease in long-term potentiation in the CA1 area of the hippocampus (Kluge *et al*., 2008). It will be important to identify and further characterize subpopulation-specific GABAergic and peptidergic mechanisms in fear memory formation and associated network activities.

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List of abbreviations.

5-HT	Serotonin
5-HT1A	Serotonin1A receptor
ACh	Acetylcholine
AKAP	A-kinase-anchoring protein
AMPA	a-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AMPAR	AMPA receptor
BLA	Basolateral amygdala
BNST	Bed nucleus of stria terminalis
CA	Cornu Ammonis
CamKII	Calcium / calmodulin-dependent protein kinase II
CamKIV	Calcium / calmodulin-dependent protein kinase IV
cAMP	cyclic Adenosine monophosphate
CBP	CREB binding protein
CCK	Cholecystokinin
cDNA	complementary DNA
CeA	Central nucleus of the amygdala
CR	Conditioned response
CREB	Calcium response element binding protein
CRF	Corticotropin-releasing factor
CS	Conditional stimulus
D1 and D2	Dopamine receptors type 1 and 2
DA	Dopamine
DG	Dentate gyrus
DH	Dorsal hippocampus
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EC	Entorhinal cortex
EEG	Electroencephalogram
EPN	Endopiriform nucleus
FF	Fimbria-fornix
fMRI	Functional magnetic resonance imaging
GABA	γ-amino butyric acid

GAD	Glutamic acid decarboxylase
GAT	GABA transporter
GFP	Green fluorescent protein
GluR1	Glutamate receptor subunits type 1
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine-5'-triphosphate
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPA	Hypothalamic-pituitary-adrenal axis
IPSC	Inhibitory post-synaptic currents
IPSP	Inhibitory postsynaptic potential
ISI	Inter-stimulus intervals
LA	Lateral amygdala
LH	Lateral hypothalamus
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MAP4	Microtubule-associated protein 4
MAPK	Mitogen-activated protein kinase
MOBP	Myelin-associated oligodendrocytic basic protein
mPFC	medial Prefrontal cortex
mRNA	messenger Ribonucleic acid
NCAM	Neural cell adhesion molecule
NGFI	Nerve growth factor-inducible
NMDA	N-methyl-D-aspartic acid
NPS	Neuropeptide S
NPSR	Orphan G-protein coupled GPR154 receptor
NPY	Neuropeptide Y
NR2B	NMDA receptor 2B
PAG	Periaqueductal gray
PCR	Polymerase chain reaction
PET	Positron emission tomography
PKA	Protein kinase A
PKC	Protein kinase C

PLSD	Fisher's Protected Least Significant Difference
PP	Perforant pathway
PSA	Polysialic acid
PTSD	Post-traumatic stress disorders
PV	Parvalbumin
REM	Rapid eye movement
RT-PCR	Real time polymerase chain reaction
sIPSPs	spontaneous Inhibitory potentials
SPL	Sound-pressure level
SST	Somatostatin
STM	Short-term memory
US	Unconditional stimulus
VGCCs	Voltage gated calcium channels.

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(Dipl. biol. Jorge R. Bergado Acosta)

Curriculum vitae.

Personal data

Name	Jorge Ricardo Bergado Acosta
Date of birth	24 July 1976
Place of birth	Matanzas, Cuba
Citizenship	Cuban
Academic grade	Dipl. Biology
Correspondence	Institut für Biology, Deparment of Molecular Neurobiology Otto-von-Guericke-Universität Magdeburg Leipziger Str. 44, D-39120 Magdeburg Tel. 0391-6755111 Email. bergado358@yahoo.com
Privat address	Acker str. 18 D-39112 Magdeburg. Germany

Education

July 1994	Graduation, High School Matanza, Cuba
Aug.1994-Aug.1995	Military service, Cardena, Cuba
Sept.1995-July 2000	Undergraduate studies (Biology) at Havana University
Sept.1999-July 2000	Diploma research studies at the Laboratory of Biomodels. International Center of Neurologic Restoration (Ciren) Havana, Cuba. Under supervision Dr. Liliana Francis Title: "Effects of the nerve growth factor (NGF) in the behavioral characterization of Huntington models induced by quinolinic acid (QA) in rats"
Sept.2000-July 2003	Museum of Natural Science. "Felipe Poey". Havana. Cuba.
Oct.2003-April 2008	Institute for Phisiology. Otto-von-Geriecke University. Magdeburg. Germany.
Since May 2008	Institute for Biology. Deparment of Molecular Neurobiology

Magdeburg, July 2009

(Dipl. biol. Jorge R. Bergado Acosta)

List of publications.

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Erklärung.

Ich erkläre, dass ich die der Naturwissenschaftlichen Fakultät der Otto-von-Guericke-Universität zur Promotion eingereichte Dissertation mit dem Titel

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im Institut für Biologie der Otto-von-Guericke-Universität Magdeburg mit Betreuung durch Prof. Dr. rer. nat. Oliver Stork ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe. Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden. Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Naturwissenschaftlichen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

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(Dipl. biol. Jorge R. Bergado Acosta)